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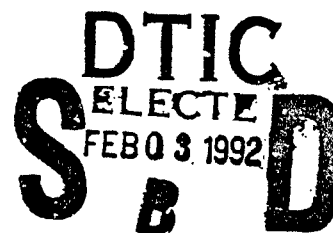
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AGENTS WHICH MEDiate PULMONARY EDEMA

Final Report

Frederick Glauser, M.D.

December, 1990



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SUMMARY

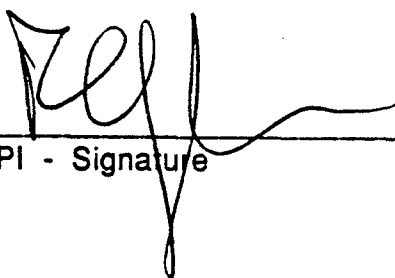
The work described in this report has been focused on delineating various approaches to understanding mechanisms of pathological changes leading to pulmonary edema. Baseline parameters including hemodynamics, gas exchange and lymph flow were determined for the sheep lung fistula model. The technique for nasotracheal intubation in awake sheep was established. Bronchoalveolar lavage (BAL) was performed on 100 sheep, and alveolar macrophages (AM), obtained by this method, were used for baseline studies of chemotaxis, phagocytosis, superoxide anion production, lysosomal enzyme and elastase release, and intracellular calcium concentrations (using fura-2). Baseline studies on tumor necrosis factor release and calcium (using ^{45}Ca) are progressing. Isolated sheep lung perfusion studies were unsuccessful and instead isolated rat lung was used for perfusion studies. Baseline studies completed in this system include permeability characteristics such as pulmonary vascular pressure and albumin uptake. Permeability of the alveolar capillary membrane in sheep was assessed by analysis of protein in BAL and demonstrated successful use of this technique. Analysis of arachidonate metabolites in BAL by high performance liquid chromatography is under progress, and results on leukotriene B₄ levels are presented. Finally, studies on pulmonary endothelium have proceeded in four areas: (1) establishment of endothelial cell lines, (2) assessment of cytotoxicity toward endothelial cells by lymph, lymphocytes and neutrophils, (3) adherence of neutrophils to endothelium, and (4) permeability of endothelial monolayers.



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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).


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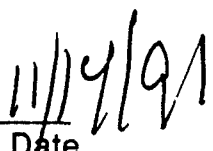

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INTRODUCTION

The mechanisms by which the development of pulmonary edema occurs are as yet poorly understood. Pulmonary dysfunction is manifested in response to many vesicants and gaseous agents encountered by military personnel. The aim of this work is to employ several approaches to understanding the mechanisms involved in the development of pulmonary edema.

To better understand the pathophysiological consequences of vesicant inhalation on the lung, we employed the sheep lung lymph fistula model (1). This "chronic" animal model employs awake sheep in which the caudal mediastinal lymph node is catheterized permitting frequent sampling of lung lymph. This model permits the monitoring of changes in pulmonary vascular endothelial permeability, systemic and central hemodynamics, and gas exchange. These changes can be monitored before and after various insults, such as endotoxin or bacterial infusion (2-4), ethchlorvynol injection (5,6), complement and leukocyte activation (7,8), pulmonary air and fibrin emboli (9-11), exposure to various toxic drugs (12,13), and interleukin-2 infusion (14).

In particular, bronchoalveolar lavage (BAL) has been demonstrated to be a powerful diagnostic and experimental tool (15). Analysis of BAL can reveal the presence of inflammatory mediators such as arachidonic acid metabolites and the infiltration of cells such as neutrophils which may contribute to inflammation and enhanced endothelial permeability which may lead to edema. In addition, analysis of alveolar macrophages (AM) from BAL may reveal contributions of this immune cell to pathological changes. The AM performs many protective functions in the alveolar space of the lung, but many of these functions, including release of lysosomal enzymes and production of oxygen free radicals, may lead to pathological changes when stimulated excessively or inappropriately.

Lavage fluid may also be used *in vitro* to assess directly the effect of mediators in the lavage on pulmonary vascular endothelium using endothelial cell line methods, as well as isolated perfused lungs. These models allow direct measurement of changes in permeability which cannot be made in the intact animal. Taken together, these studies will provide important insights into the physiological mechanisms which contribute to

the development of pulmonary edema, as well as possible mechanisms for intervention and/or protection from pathological changes.

MATERIALS AND METHODS

Bronchoalveolar Lavage: The lavage procedure was performed through an indwelling endotracheal tube following proper general anesthesia (Pentobarbital 25-30 mg/kg). Once the endotracheal tube was placed, the animal was mechanically ventilated (Harvard Animal Ventilator) with supplemental O₂ added to maintain stable arterial oxygen and carbon dioxide levels. A 4 mm fiberoptic bronchoscope (Olympus Corp.) was visually guided through the tracheobronchal tree and gently wedged into a segmental orifice of the right lower lobe. Fifty milliliter aliquots of sterile 0.9% NaCl were instilled through the bronchoscope and gently aspirated back into the injecting syringe. A total volume of 300 ml was used for the procedure. The recovered lavage samples were immediately placed at 4°C and were kept at that temperature until processing.

Chemotaxis: Using the technique of Smith et al. (16), chemotaxis of sheep alveolar macrophages (SAM) in modified blind well Boyden chambers was tested against N-formyl methionine leucine phenylalanine (FMLP, Sigma Laboratories, St. Louis, MO) and phorbol, 12-myristate, 13-acetate (PMA, LC Services Co., Woburn, MA). The results are determined by counting the number of macrophages that have migrated completely through the filter in 20 oil immersion fields (OIF) per filter and were expressed as the mean + S.E.M. of three filters. Any contaminating cells (i.e., PMN or eosinophils) which migrated through the filter were not counted.

Lysosomal Enzyme Release: The technique of Fowler et al. (17) was modified to determine the amount of N-acetyl- β -D-glucosaminidase released by SAM. The SAM were resuspended in Gey's balanced salt solution (GBSS) containing 0.2% bovine serum albumin (BSA) (fraction V) to a concentration of 1×10^6 /ml, and 0.45 ml of the cell suspension was added to each experimental tube. Either 0.05 ml of control (medium), vehicle (0.1% dimethylsulfoxide (DMSO)), or secretagogue (100 nM PMA or 1 μ M A23187) was added to the cells. This mixture was then incubated at 37°C for one hour, after which the cells were centrifuged (400 x g, 10 min). The supernatants were aspirated and placed in fresh tubes at 4°C. The cell pellets were disrupted by a 10 min incubation with 0.1% Triton X-100. The lysed pellets were then centrifuged (400 x g, 10 min) and the supernatants were aspirated, and placed in fresh tubes. The substrate, N-

acetyl- β -D-glucosaminide, was added to all the tubes, which were then incubated at 37°C for 30 min. The reaction was stopped by the addition of 25% trichloroacetic acid (TCA); the samples were then centrifuged at 1000 x g for 30 min. Ammonium hydroxide (11.7%) was added to this final supernatant and absorbance determined spectrophotometrically at 410 nm (Beckman Model 34). The results are expressed as the percentage of total enzyme released using the following formula:

$$\% \text{ Release} = \frac{\text{Enzyme in the supernatant}}{\text{Enzyme in supernatant} + \text{enzyme in pellet}} \times 100$$

Elastase: The activity of the elastin degrading enzyme (i.e., elastase), in AM supernatants was measured by the method of Del Mar et al., (18) and Nakajima et al. (19). This method utilizes a peptide substrate coupled to nitroanilide (NA), methoxy-Suc-Ala-Ala-Pro-Val-p-Na (Sigma Chemical Co.). This has been shown to be an excellent substrate for human leukocyte elastase and a poor substrate for other proteases such as cathepsin G (19). Elastase cleavage of the peptide released the NA group, and an increase in absorbance at 410 nm was subsequently measured (20). The substrate solution contains 2.5 mM substrate in 0.1 M HEPES, pH 7.5, 0.5 M NaCl, 10% dimethylsulfoxide (DMSO); 25 μ l of test solution was added to 1.8 ml of substrate solution and absorbance at 410 nm was monitored continuously for 2 - 60 min. Test solutions consisted of supernatants from SAM plated at 106/well in 24-well plates.

Superoxide Anion Production: Using the methods of Johnston et al. and Joseph et al. (21,22), the reduction of ferricytochrome C was followed as an indicator of superoxide anion production in SAM. SAM (1×10^6 per well) was plated in 24-well tissue culture plates (Costar) and adhered for 60 minutes at 37°C, 5% CO₂. The cells were washed once with RPMI 1640 medium and then once with Hanks' Balanced Salt Solution (HBSS). HBSS containing cytochrome C (80 μ M) + superoxide dismutase (SOD, 10 mg/ml) was then added to the cells (1 ml/well). PMA (5 μ M) was added to the above mixture when studies were performed on stimulation of the baseline response. Following a 90 minute incubation at 37°C, all supernatants were aspirated, centrifuged at 8000 x g for two minutes, and then the absorbance of the supernatant determined at 550 nm (Beckman Model 34). The data are expressed as nanomoles of O₂⁻ produced.

Phagocytosis: Phagocytic function of SAM was measured after the method of Goodell et al. (23) and Kessler et al. (24). For these experiments, antibody was raised in guinea pigs as described previously

(23). The data are expressed as the number of counts internalized per 1×10^6 SAM.

Calcium Fluxes: Fura-2 Fluorescence: Fura-2 fluorescence was measured according to the methods of Grynkiewicz et al. (25) and Tsien et al. (26). Sheep AM were washed and suspended in saline buffer (145 mM NaCl, 5 mM NaH_2PO_4 , 1 mM CaCl_2 , 0.5 mM MgSO_4 , 5mM glucose, 10 mM HEPES, pH 7.4) and then loaded with 1 mM fura-2 methyl ester for 60 minutes. SAM were then washed and resuspended in fresh saline buffer and stimulated with PMA (10^{-6}M), Ionomycin (10^{-6}M), A23187 (10^{-6}M), and FMLP (10^{-8}M), prior to being placed in position on a Spex dual wavelength spectrofluorometer. Fluorescence intensity was read simultaneously at 340 and 380 nm excitation and the ratio of these readings was used to calculate free ionized calcium based on calibration curves (505 nm emission). Calibration curves for fluorescence patterns were generated using saline buffer containing known concentrations of calcium, controlled by Ca-EGTA buffer assuming dissociation constants for Ca-EGTA complex of 380 nM in this buffer.

Tumor Necrosis Factor - L929 Bioassay: The L929 bioassay developed by Flick and Gifford (27) and then modified by Mukavitz-Kramer and Carver (28) and Denizot and Lang (29) was used to detect the presence of TNF from SAM. On day 0, the SAM were purified and plated at 7.5×10^5 cells/well of a 24-well plate. After adhering for 60 min, 39°C , the media was decanted and the appropriate stimuli added to the cells (PMA 10^{-9} - 10^{-6}M , FMLP 10^{-9} - 10^{-7}M , or LPS 20 - 200 ng/ml). The SAM + stimulus were returned to the incubator and kept at 39°C for 24 hrs. Also, on day 0, L929, a murine tumorigenic fibroblast (ATCC, CCL1) were plated at 4×10^4 cells/well of a 96-well plate and also incubated for 24 hrs at 37°C .

The next day, (Day 1), the spent media from the L929 cells was decanted and replaced with fresh medium + actinomycin D (2 mg/ml). The supernatants from the SAM \pm stimuli were removed and added to the L929 cells + Actinomycin D. After incubating 20 hrs at 37°C , (Day 2), the spent media was carefully removed and crystal violet (0.5%) added to all the wells. The plates were incubated at 37°C , 10 min and vigorously rinsed with distilled water. The plates were read at 570 nm, using a Biorad EIA Reader (Richmond, CA). The data are expressed as % cytotoxicity using the following calculation:

$$\% \text{ cytotoxicity} = \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100$$

Determining the Cytolytic Potential of Vesicant Exposure on Pulmonary Artery: Our aim in these experiments was to determine whether direct contact between endothelial cells and agent (Phorbol Myristate Acetate), or humoral substances secreted by host effector cells in response to agent exposure (PMA), results in endothelial cell killing. In addition, we tested effector cells to determine whether agent exposure (in vivo and in vitro) induced nonspecific cytotoxic activation. The release of ^{51}Cr from labeled endothelial cells was studied to determine viability of endothelial cells when challenged by the experimental condition. When host effector cells were investigated, they were incubated with the endothelial cell targets at varying effector to target ratios. Varying fractions of the LD₅₀ of the agent or concentrations of the biological fluids were incubated with the endothelial cells to assess their cytotoxic potential.

Endothelial cells, at semi-confluence in 25 cm² flasks, were incubated in DMEM with 10% FBS in the presence of 200 uCi of fresh ^{51}Cr for 18 hours. Monolayers were washed 3 times in fresh DMEM with 10% FBS and gently rocked to remove free label. Fresh medium was added and the labeled cells were allowed to incubate at 37°C for an additional 30 min. The flasks were washed with fresh medium as above. The cells were removed from flasks by trypsinization (EDTA-Trypsin, GIBCO), suspended in DMEM without serum (serum free medium, SFM) and counted. Five thousand labeled endothelial cell targets were added to the wells of a 96-well culture plate. The agent was added at a solution of varying fractions of the LD₅₀ and the plate was incubated at 37°C for 4 hours. Control wells of endothelial cells plus medium/diluent were established. All experiments were performed in triplicate. At the end of the incubation period, the supernatants were harvested using a supernatant harvester (Skatron) and the gamma activity was determined. Maximum isotope release was measured by incubation of the targets with 0.1 N HCl. Spontaneous release was measured by incubation of the targets in culture medium/diluent without challenge. Specific cytotoxicity was calculated by:

$$\frac{\text{Experimental Counts} - \text{Spontaneous Release}}{\text{Total Lysate Counts} - \text{Spontaneous Release}} \times 100$$

RESULTS AND DISCUSSION

Sheep Model: Results have been obtained using the awake sheep model in three general areas over the last 18 months: 1) bronchoalveolar lavage, 2) baseline measurements of hemodynamic, gas exchange and lung lymph flow in awake, unanesthetized control sheep, and 3) techniques for naso-tracheal intubation in the awake sheep.

Bronchoalveolar Lavage (BAL) - During the last 18 months approximately 100 BALs have been performed in a similar number of sheep. Two techniques have been employed: 1) 35 sheep were lightly anesthetized with intravenous sodium amytal (30 mg/kg) and orally intubated with a # 8 F Shiley low pressure cuffed endotracheal tube. Animals were placed in the supine position on a surgical table and given supplemental oxygen. A specially designed extra-long Mashita fiberoptic bronchoscope (FOB) originally designed for large animals was used to perform BAL. The FOB was passed through the endotracheal tube into the right middle lobe and the tip wedged. Fifty ml of sterile 0.9% NaCl was infused slowly into the segment and removed by gently suctioning. This procedure was repeated three times. The bronchoscope was then placed in the left lower lobe and the above procedure repeated. The BAL samples were immediately placed on ice in 50 ml polyvinyl tubes and capped. The total amount of fluid lavaged and the return was noted. These samples were then sent on ice to Dr. Caroline Kramer's laboratory at the Medical College of Virginia for further processing. 2) In approximately 65 sheep, the above procedure was modified as follows: following the placement of the oral endotracheal tube, a tracheostomy was performed and a # 8 Shiley endotracheal tube inserted. This procedural change was necessary while the Mashita bronchoscope was under repairs, since shorter bronchoscopes cannot be wedged through an orally inserted endotracheal tube. The above procedures for BAL were then performed and samples handled as above. There was no difference in the amount of fluid infused or the lavage returned using this procedure, suggesting that both procedures are comparable.

Chronic Sheep Lung Lymph Fistula: The chronic sheep lung lymph fistula model is uniquely adapted for following cardiopulmonary hemodynamics, gas exchange, pulmonary microvascular permeability, and blood and BAL cellular changes over time following either intravenous or inhaled exposures. The technique for inserting the lymph catheter is as follows:

On day 1, the sheep is anesthetized with 30 mg/kg of Surital IV, orally intubated, ventilated with a FiO_2 .5, Vt 10 - 15 ml/kg, and a RR of 10 - 12/min. Permanent carotid artery and a jugular venous catheter are inserted percutaneously. The animal is allowed to awaken and taken back to its pen. On day 2, the animal is reanesthetized, intubated, and ventilated on an FiO_2 1.0 at the ventilatory settings. A right sided thoracotomy is performed between the 5th and 6th intercostal space and the caudal mediastinal lymph node and its duct are isolated and cannulated with silastic tubing. This catheter is stabilized by suturing it to the interior chest wall, an incision made between the 6th and 7th intercostal space, and the catheter is exteriorized. A suture is placed securely around the caudal mediastinal lymph node approximately one-third of the way from the caudal end. Diaphragmatic lymphatics are cauterized. The thoracotomy incision is sutured and the externalized silastic tubing is secured to the skin. The animals are allowed to awaken and are returned to their pens.

The lymph is usually free of any blood by postoperative day 2 to 3. The animals are then assigned to be studied if they meet the following criteria: 1) mean systemic blood pressure > 65 - 70 mmHg, 2) PaO_2 > 85 - 90 torr on FiO_2 0.21, 3) mean pulmonary artery pressure of < 20 mmHg, 4) stable, blood free, lymph flow.

Approximately 75% of animals will have successful implantation of the silastic catheter into the caudal mediastinal lymph node. Approximately 50% of all animals will have continuous lymph flow for greater than 3 days and meet the above criteria for entering into the study.

The results of baseline, control measurements taken from 10 sheep can be found in Tables 1 and 2. All values indicated are within the normal range.

Endotracheal Intubation - In 3 awake sheep we attempted endotracheal nasal intubation with a # 7.5 endotracheal tube over the long fiberoptic bronchoscope. Although we were eventually successful in performing this technique, we were unhappy with the results for the following reasons: 1) animals required 10 - 20 mg of valium to keep them calm enough to perform the procedure, 2) trauma was easily induced as evidenced by increasing secretions and by bleeding from the trachea, 3) the time to successful endotracheal intubation was approximately 10 mins with multiple attempts. For the above reasons we have changed our

technique to the following: 1) animals are lightly anesthetized with 30 mg/kg sodium amytal and a #7.5 - 8 F polyvinyl endotracheal tube is inserted orally and a bite block placed securely. The animal is then allowed to awaken, placed in his cage and a loose head restraint attached.

Sheep Alveolar Macrophages: Alveolar macrophages (AM) were obtained from bronchoalveolar lavage (BAL) as described above. The recovered BAL fluid represented greater than 60% of the instilled volume and typically contained 25 to 30 x 10⁶ cells. Differential cell counts performed immediately after lavage revealed 85% AM, 11% lymphocytes and 4% neutrophils with little erythrocyte contamination. Viability as determined by trypan blue dye exclusion was greater than 95%. The BAL was centrifuged (400 x g, 10 min), supernatant frozen for later use, and the cell pellet purified using Sepracell. Cells purified by this technique were >95% AM with no decrease in viability. These cells were used in the following assays: phagocytosis, superoxide anion (SOA) production, lysosomal enzyme release (LER), intracellular calcium concentration, elastase release and tumor necrosis factor (TNF) release.

Phagocytosis: Phagocytosis of inhaled particles and bacteria is a critically important function of the AM. This ability was measured as described in Methods. Phagocytosis of opsonized chromated sheep erythrocytes at 37°C by sheep AM obtained on day 1, 2, or 3 of subsequent lavages of the same sheep is indicated in Figure 1. On day 1, AM phagocytosis was relatively low for the first 30 min of incubation, then markedly increased in rate, with a peak at 60 minutes and remained elevated at nearly double baseline phagocytosis for the 180 min. time course measured. In contrast, on days 2 and 3, the rate of phagocytosis was much slower, although on day 2 the amount of particles phagocytized at 180 min. was equivalent to the same endpoint as on day 1. Thus, at 37°C, AM obtained by successive lavage may have a slower rate of phagocytosis but relatively unimpaired capacity to phagocytize.

In Figure 2, similar results are shown at 39°C, which is the body temperature of the sheep. These experiments were performed to determine whether AM function may vary when incubation temperature is changed from 37°C (standard procedure) to 39°C, which is physiological for sheep. As shown in this figure, both the rate and magnitude of phagocytosis was enhanced on day 3 at 39°C vs. 37°C. However, on days 1 and 2, there was little difference between 37°C and 39°C. At 39°C, phagocytic rate and the amount of particles phagocytized over the 180 min time course were depressed on days 1 and 2 as compared to day 3.

Superoxide Anion: Production of oxygen radicals such as superoxide anion (SOA) is triggered in AM during immune or inflammatory responses and is an important bactericidal mechanism employed by the AM. In Figures 3 and 4, production of SOA (as measured by ferricytochrome C reduction) by AM is depicted. These studies utilized the same approach as described above for phagocytosis; i.e., comparisons were made at 37 vs 39°C and on days 1, 2 and 3 of successive BALs. In Figure 3, the concentration response curve for SOA production by the stimulant phorbol 12-myristate, 13-acetate (PMA) is depicted. Baseline SOA production in sheep AM was similar to that observed previously in guinea pig AM. Stimulation by PMA was relatively modest, 3 to 5-fold over baseline, and was dose related from 10^{-8}M to 10^{-6}M PMA on days 1 and 2. There was little difference in the response on day 1 vs. day 2 of lavage. In contrast, on day 3, there was a marked enhancement in SOA production, which was dose-related from 10^{-9}M to 10^{-6}M PMA, and reached over 10-fold stimulation over baseline. Thus, on day 3 of lavage, AM appear to be primed for SOA production by PMA.

Figure 4 depicts studies performed in parallel with those in Figure 3, but at 39°C. There are no apparent differences in the SOA response profile at 37°C vs. 39°C, in contrast to results with phagocytosis. For all subsequent studies, experiments were performed on cells obtained on day 1 of lavage, which exhibit optimal phagocytosis and probably represent a more typical resting AM.

Chemotaxis: Chemotaxis, or directed cell movement, is triggered during inflammation or infection in order to recruit AM to the site. Impairment of this function may contribute to reduced host resistance. Figure 5 illustrates the chemotactic response of sheep AM to the chemoattractant PMA at 37°C and 39°C. These experiments were performed according to standard procedures using Boyden chambers as described in Methods. At both temperatures, a dose-response relationship is seen, with a peak chemotactic response at 10^{-8}M PMA. This was the same optimal concentration for this response in guinea pig AM. Unlike the phagocytic function, chemotaxis was unaffected by temperature. Therefore, 39°C was used for all subsequent experiments, since this temperature is physiological for sheep cells and phagocytic ability was optimal at 39°C.

Lysosomal Enzyme Release: Macrophages contain abundant lysosomal enzymes which allow this cell to digest phagocytized particles. Some enzyme release occurs during phagocytosis although the role of lysosomal

enzyme release (LER) in normal functioning of the macrophage is unclear. However, reduced levels of these enzymes or altered LER may lead to reduced clearance ability of AM or, if stimulated excessively, to damage to the host tissues. Release of the lysosomal enzyme N-acetyl- β -D-glucosaminidase (NAGA) is depicted in Figure 6. Sheep AM were plated at 1×10^6 cells/well in a 24-well plate, allowed to adhere for 60 min at 39°C, then washed and stimulated for 60 min or 24 hours at 39°C. Opsonized zymosan (OpZ), a yeast particle which has been coated with human serum, was used as a stimulus. As shown in Figure 6, stimulation with OpZ for 60 min did not stimulate release of NAGA; however, after 24 hour stimulation, 1-4 mg/ml OpZ produced 45-50% release of NAGA as compared to the baseline release of 5%. This response was reproducible but not dose-related. In order to assess any role of the Fc receptor and/or complement pathway in the LER response to OpZ, zymosan was also studied. The LER response to zymosan was highly variable, ranging from no stimulation above baseline to a stimulation of LER equivalent to that evoked by OpZ in the same dose range. Although these experiments were repeated 5 times, we feel that due to the variability these results are uninterpretable and the data has not been included as a figure in this report.

Several other agents which had been shown to enhance LER in other cells were used unsuccessfully as stimuli for LER in sheep AM. PMA (10^{-6} to 10^{-11} M), the synthetic peptide, FMLP (10^{-6} to 10^{-10} M), the calcium ionophore A23187 (10^{-6} to 10^{-11} M), and various combinations of A23187 + PMA and A23187 + FMLP did not enhance LER under any of the following conditions: 1 hour adherence, 1-3 hour stimulation; 1 hour adherence, 24 hour stimulation; 24 hour adherence, 1-3 hour stimulation (data not shown). Since, under some of these conditions, LER was enhanced by OpZ, we can conclude that while sheep AM can release lysosomal enzymes, this response is not enhanced by many stimuli which cause enhanced LER in other cell types.

Calcium Concentration (Fura-2): Measurement of free intracellular calcium concentration was chosen as a biochemical correlate of alveolar macrophage function. Calcium has been shown to act as a signal transducing mechanism in many responses of the macrophage (30); thus, alterations in calcium concentration or calcium metabolism may lead to deficits in function or to inappropriate macrophage stimulation.

Results from studies of intracellular calcium concentrations in the sheep AM (SAM) are presented in Figures 7-12. Cells were obtained and

purified according to standard operating procedures, then were adjusted to 5×10^6 cells/ml in Gey's Balanced Salt Solution (GBSS). SAM were loaded with 1 μ M fura-2-acetoxymethyl-ester in a rotator at 37°C for 1 hour, then transferred in 1 ml aliquots to microfuge tubes on ice. This procedure did not affect cell viability as determined by trypan blue dye exclusion. At the time of the assay, an aliquot of cells was centrifuged and resuspended in 2 ml fresh GBSS at 37°C, then placed in a stirring cuvette in a Spex dual beam spectrofluorometer. Fluorescence was measured simultaneously at 340 nm (calcium-bound fura-2) and 380 nm (calcium-free fura-2). This data is shown in Figures 7, 9 and 11. The ratio of 340/380 fluorescence was determined, to be used later for calculation of calcium concentrations, and this data is shown in Figures 8, 10 and 12. Each of the graphs is from a representative experiment of three experiments, each performed at least in duplicate with similar results.

Figure 7 illustrates the effect of the calcium ionophore, 10^{-6} M ionomycin on the fluorescence of fura-2 at 340 and 380 nm. The downward spikes which reach the x-axis in this and other graphs represent technical artifacts which occur when the cuvette port is opened for the addition of reagents. As shown in Figure 7, addition of ionomycin resulted in a rapid increase in fluorescence of fura-2 at 340 nm, indicating a rise in free intracellular calcium concentrations. This response is different from that seen in our laboratory using guinea pig AM, or in other laboratories using lymphocytes or neutrophils, in which a sharp peak in calcium concentrations followed by a gradual return to baseline is seen. In the SAM, ionomycin caused a rapid, sustained elevation in calcium concentration. After a five minute period of stimulation, triton X-100 (10%) was added to lyse the cells and to then be able to obtain the fluorescence maximum for these cells. EGTA (0.25M) was then added to chelate calcium for determination of the fluorescence minimum. These figures will be used to convert fluorescence values to calcium concentrations; the computer system used for this is currently under repair. Those results will be presented in a subsequent report.

Figure 8 represents the conversion of Figure 7 to ratio form, which will later be converted to calcium concentrations. The peak seen at approximately 280 seconds is due to the drop in 380 nm fluorescence seen in Figure 7; we are not yet certain of the significance of this peak, if any. However, the same peak is seen in each of Figures 7 through 12, and therefore most likely represents a technical artifact.

Figure 9 illustrates the rise in fura-2 fluorescence in SAM treated with 10^{-7} M FMLP. These data are converted to ratio form in Figure 10. FMLP caused a very slight, but like ionomycin, rapid and persistent elevation in calcium. The typical response to FMLP in other cell types is similar to that of calcium ionophore, but smaller in magnitude; therefore, this response to FMLP is unusual. This effect of FMLP was observed consistently in replicate samples and in separate experiments.

Figure 11 illustrates the effect of 10^{-6} M PMA on fura-2 fluorescence. PMA had no effect on calcium concentrations. This sample was also used for determination of fluorescence maxima and minima. Figure 12 represents the conversion of Figure 11 to ratio form.

These results demonstrate that fura-2 can be used for determination of intracellular calcium concentrations in SAM without affecting cell viability, and suggest that ionomycin and FMLP cause a rapid and persistent elevation of calcium concentrations which is not typical of the response seen in other cell types.

Elastase: The release of lysosomal enzymes from the PMN and the alveolar macrophage has been shown to be involved in connective tissue destruction associated with certain diseases such as emphysema (31) and atherosclerosis (32). Both these cell types contain abundant quantities of the enzymes collagenase and elastase which are extruded during phagocytosis and may subsequently contribute to lung damage (33-35). The presence of elastase in the alveolar macrophage has been demonstrated in a variety of animal models including rat (36) and guinea pig (34). During these studies we had two primary objectives: 1) using the spectrophotometric techniques of Del Mar et al. (18) and Nakajima et al. (19) to establish the presence of elastase in SAM and 2) quantify the amount of enzyme present in the SAM.

Using the same parameters that we had established for the measurement of N-acetyl- β -D-glucosaminidase, initial experiments with SAM did not detect any elastase. A variety of conditions were manipulated in order to optimize the assay without success. The modifications included: 1) changes in cell number ($0.5 - 2 \times 10^6$ cells/well); 2) changes in the incubation time (1 - 24 hours); 3) decreasing the volume of media in the well so that we were assaying a larger percentage of available enzyme; 4) changing the lysing agent from 0.05% Triton X-100 to ammonium chloride; 5) allowing the plate to develop longer after the addition of substrate (1 - 72 hours); and 6)

testing PMA, FMLP, A23187 and OpZ as stimuli. A total of seven experiments were performed incorporating the conditions delineated above without any reproducible detection of elastase in SAM.

In order to ascertain the sensitivity of the technique, purified elastase from porcine pancreas was ordered and a standard curve determined from 0.05 $\mu\text{g/ml}$ to 0.3 $\mu\text{g/ml}$. Within 2 minutes of the addition of the substrate (meo-suc-ala-ala-pro-val-p-NA) measurable amounts of elastase were recorded and a time course followed for 10 min (data not shown).

Because the PMN is also recognized as containing large quantities of elastase, we measured the elastase found in human PMNs (HPMN) as a positive control for our assay. The results from these experiments are shown in Fig. 13. Even non-stimulated HPMN (control) demonstrated measurable amounts of enzyme 5 minutes after the addition of substrate. From Fig. 13, it appears that there were decreasing levels of elastase over time. As seen in Fig 14, what was actually occurring was the rapid intensification of the amount of color seen in the pellet fraction over 35 min. vs. the much smaller changes seen in the supernatant fraction. Since the % elastase released is a ratio of the supernatant and pellet values, it incorrectly appears that enzyme levels were decreasing. It should be noted that 10^{-8}M PMA was a potent stimulator of elastase in HPMN unlike the SAM.

Having been unable to measure elastase using up to 2.5×10^6 SAM/well, the decision was made to vary the cell number up to 15×10^6 SAM to determine when we could detect the presence of the elastase enzyme. Aliquots of $1.5 - 15 \times 10^6$ cells were lysed using 0.05% Triton X-100 and the cell fragments centrifuged out at $400 \times g$ for 10 min. The supernatants were combined with substrate and the development of the enzyme followed over time (5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min.). Fig 15 clearly indicates that not until we used 5×10^6 cells/sample were we able to detect any enzyme. The control was 0.05% Triton X-100.

With regards to the stated objectives, we were able to demonstrate through the standard curve using purified elastase, the hPMN studies, and the cell titration curves that the technique of Del Mar et al. and Nakajima et al. (18,19) detect the presence of elastase. However, based on the data in the cell titration curves, (Fig. 15), the levels of elastase found in the

SAM are so low as to make it impractical to attempt to perform this assay on a routine basis.

Tumor Necrosis Factor: Tumor necrosis factor (TNF), also known as cachectin, has been associated with a wide array of biological activities integral to an acute or chronic inflammatory state (37-39). In addition, TNF has been identified as being part of the non-specific anti-viral and anti-neoplastic system in both human and animal models (40-42).

Activated human blood monocytes (43), various animal macrophage cell lines (44,45), as well as alveolar macrophages (46) have all been recognized as being capable of synthesizing and releasing TNF. Because it is now accepted that mononuclear phagocytes serves as a major source of TNF (37-39) and that TNF works as a major mechanism by which macrophages attack and/or destroy tumor cells (37-41), we were interested as to whether SAM would express and release TNF in the surrounding milieu.

Using human recombinant TNF (rTNF), our first experiments were designed to establish a standard curve using L929 cells. Fig. 16 shows these results with concentrations of 0.01 U/ml - 3.0 U/ml of rTNF. This graph is a representative one chosen from 9 experiments. Based on the data in Fig 16, 2 U/ml of rTNF results in 50% cytotoxicity.

Lipopolysaccharide (LPS) has been demonstrated to stimulate the release of TNF from peripheral blood monocytes (47). LPS (20 - 200 ng/ml) was incubated (39°C) with adherent SAM for 24 hrs. The supernatants from LPS-stimulated SAM were then added to adherent L929 and incubated for another 24 hrs. SAM viability was assessed after 24 hrs with the LPS using trypan blue dye exclusion. At 200 ng/ml of LPS, there was a small decrease in SAM viability as compared to the other concentrations of LPS (data not shown). The results from the LPS experiments are shown in Fig 17. Because the TNF response to LPS stimulation did not appear highly sensitive, as indicated by the absence of a dose response relationship, both PMA and FMLP were studied as potential stimulators of TNF. FMLP (10^{-9} - 10^{-7} M), like LPS, did not appear to stimulate TNF production (Fig 18). However, the 24 hr stimulation of SAM with PMA (10^{-9} - 10^{-6} M) resulted in a dose-related increase of TNF that was substantially greater than that induced by either LPS or FMLP (Fig 18).

Sheep Lung Perfusions: Seven attempts were made to isolate and perfuse sheep lungs. None were successful. We were able to isolate the appropriate vascular connections and to establish circulation and ventilation, but none of the preparations became stable. The initial vascular pressures were low (pulmonary artery = 12.1 ± 1.0 mmHg and pulmonary vein or left atrial pressure 3.7 ± 0.4 mmHg) but increased rapidly (approx. 1.0 mmHg/min) after perfusion was started and pulmonary edema was evident within one half hour. Airway pressures (14.5 ± 1.2 cmH₂O) followed a similar course and increased to 29.4 ± 6.7 cmH₂O. We considered a number of reasons for our poor results including: a) long surgical preparation time, b) introduction of air bubbles or cellular aggregates in the perfusate, c) poor oxygenation or perfusion during the procedure, and d) a perfusate electrolyte abnormality. We made several modifications in our technique to correct these potential problems but the results were always the same. We contacted several other laboratories and they experienced similar difficulties with sheep lungs although at least one laboratory has been successful.

Rat Lung Perfusions: Our difficulties with the sheep perfusions forced us to consider alternatives. An isolated, ventilated and perfused rat lung offered numerous advantages and an outline of proposed experiments was approved for use in this contract work. Our technique is detailed below.

Rats are anesthetized with methoxyflurane and thiamylal sodium. A tracheostomy is performed and the animals ventilated with room air, 2.5 ml tidal volume at 80 breaths/minute and 2 cm H₂O positive end-expiratory pressure. The pulmonary artery is cannulated through an incision in the right ventricle. Perfusion (0.02 ml/g body weight) is started with a modified Krebs-Henseleit buffer perfusate containing 20 ml N-2-hydroxyethyl-piperazine-2-ethanesulfonic acid buffer (HEPES) (pH 7.4) and 0.02 g/ml bovine serum albumin (fraction V, Sigma chemical, St. Louis, MO). A large bore cannula is secured in the left ventricle and the lung carefully removed from thorax and suspended from a ligature on a force transducer. Pulmonary perfusate flow is increased to 14 ml/min (0.07 ml/g body weight) and left atrial pressure adjusted to 4 cm H₂O by adjusting the height of the left ventricular catheter. To reverse any atelectasis the lung is gently inflated three times to 25 cm H₂O and allowed to deflate to 2 cm H₂O before resuming ventilation. When the draining effluent is clear of blood, the volume in the perfusate reservoir is adjusted to 40 ml and recirculation is established.

Pulmonary arterial (Ppa) and left atrial pressures are measured by a pressure transducer positioned at the height of the lung apex and continuously recorded on a strip chart recorder. Pulmonary capillary pressure (Ppc) is estimated using the double-occlusion method. Weight change is measured by a force transducer and recorded continuously.

Using this isolated lung preparation, we are able to evaluate the permeability characteristics of the lung in several ways including:

- 1) wet/dry weight ratios, a measure of pulmonary edema
- 2) ¹²⁵I-albumin accumulation, a measure of vascular permeability
- 3) thromboxane B₂ determination, a product often found with acute lung injury.

The techniques we used to measure each of these are described briefly below.

Pulmonary edema is measured gravimetrically as the ratio of the wet weight of the lung compared to its dry weight after the lung tissue is dried to a constant weight in a microwave oven.

Pulmonary microvascular permeability is determined by the timed accumulation of ¹²⁵I-albumin in the lung. At the conclusion of the experimental period ¹²⁵I-albumin is added to fresh perfusate at 105 cpm/ml and circulated for 3 min. ¹²⁵I-albumin exposure is terminated by perfusing with fresh unlabeled perfusate for 7 min resulting in perfusate counts <1% of the original values. The radiolabeled albumin content of the lungs and a 1 ml sample of "hot" perfusate are measured in a gamma counter adjusted for maximum ¹²⁵iodine counting efficiency. We calculate PS, or permeability surface product, but report our results as ¹²⁵I-albumin uptake because the relative contributions of permeability and surface area to albumin accumulation are unknown in spite of efforts to limit changes in surface area by creating a zone 3 lung (Ppc > PA).

Thromboxane B₂ (TxB₂) is the stable degradation product of thromboxane A₂, a product of prostaglandin metabolism often associated with lung injury. TxB₂ concentrations of perfusate effluent samples collected at the end of the 40 min perfusions are determined by the double-antibody radioimmunoassay technique using a tritiated RIA kit.

Our results in 12 normal rats using these techniques are:

Ppa = 6.7 ± 0.5 mmHg

Ppc = 4.9 0.3 mmHg
ALB. UPTAKE = 0.33×10^{-3}
W/D = 6.2 0.4
TxB2 = 20.4 3.6 pg/ml

We conclude that isolated rat lungs perfused for up to 2 hr demonstrate stable pulmonary vascular pressures, low levels of albumin uptake and TXB₂ production and normal W/D (wet/dry) weight.

Exposure Chamber: Because no exposure chamber exists in our laboratory, one will have to be assembled. Dr. Paul Fairman has contacted Dr. Lehnert at The Los Alamos National Laboratory and he forwarded a schematic diagram and photograph of the system they use for rats. With some modifications of the details, we have drafted a schematic diagram of a system suitable for our own use. With such a system, the ventilating gas could be changed to include any number of toxic gas mixtures and the resulting injury studied with the techniques described above.

Conjugated Diene Assay: The toxic agents that will be used in the experiments may lead to pulmonary injury through lipid peroxidation. We have performed preliminary measurements of perfusate and total lung conjugated dienes (a measure of lipid peroxidation) in 4 rats. These preliminary experiments have not yielded reproducible results, indicating that further experiments are needed in this area.

Permeability Characteristics and Arachidonate Metabolites in Ovine Lung: In this portion of the report we outline preliminary studies focused upon study of the alveolar-capillary membrane permeability of uninjured sheep lung and the basal production of arachidonic acid (AA) metabolites in the distal airspaces. To accomplish these studies bronchoalveolar lavage (BAL) was performed via techniques described previously in 5 animals. Briefly, male sheep weighing between 20-25 kg were anesthetized and tracheally intubated with a cuffed 8 mm internal diameter endotracheal tube. Animals were then mechanically ventilated (tidal volume 12 ml/kg) and observed for 15 minutes to insure anesthetic plane and hemodynamic stability. Animals then underwent bronchoalveolar lavage through the indwelling endotracheal tube using a specially adapted 4 mm fiberoptic bronchoscope which permitted placement in 3rd to 4th order bronchi of right middle and lower lobes. Following bronchoscope placement, 150 ml volumes were infused in 50 ml aliquots into the middle and lower lobe respectively. BAL fluid was returned by gentle suction on the injecting syringe. Syringes were immediately placed at 4°C and transported to the laboratory for analysis.

Following BAL, animals remained tracheally intubated until spontaneous ventilation was observed. Animals were then extubated and returned to the vivarium.

Bronchoalveolar Lavage Fluid Processing: BAL fluid was filtered through sterile gauze to remove particulate matter. Non-cellular fractions for protein and AA metabolite measurements were obtained by centrifugation of filtered BAL fluid for 10 minutes at 400 x g at 4°C. One ml aliquots of cell free BAL fluid were frozen (-20°C) for later analysis of total protein (see below). Analysis of cellular constituents were reported above. Fifty ml aliquots of BAL supernatants were then 5X concentrated over ultrafiltration membranes possessing 5 kD molecular weight cutoff.

Total Protein Determination: A modification of the Lowry protein method which adjusts for lipid contamination of BAL samples was used for total protein content determinations of BAL fluid. One ml cell free BAL fluid was mixed thoroughly with 3 mls of reagent solution containing 2% Na₂CO₃, 0.4% CuSO₄ 5H₂O and incubated for 10 minutes at 22°C. Folin-phenol reagent (300 µl, 1N) was then added, the sample vortexed and optical density determined by spectrophotometer (Pye-Unicam) at 600 nm. Standard curves were constructed using 5-300 µg of bovine serum albumin per sample. Figure 19 shows the results of total protein content performed on 5 BAL samples (results expressed as mean ± SEM). We found the distal airway of normal sheep to contain 456 ± 13 mg/ml of BAL fluid. Also depicted on figure 19 are the results of BAL fluid total protein content determinations performed on BAL samples obtained from 5 human subjects and 5 anesthetized pigs. These additional samples were obtained from prior studies performed in this laboratory. BAL samples from human and pig reveal that the sheep normally exhibits significantly higher basal protein content in the alveolar spaces. Also displayed on figure 19 are the results of total protein content determinations performed in 5 sheep one hour following the intravenous administration of Ethchlorvynol (ECV), a sedative hypnotic agent known to produce lung injury in humans. As indicated in figure 19, lung injured sheep exhibit significant increases in BAL total protein content above that observed in the non-injured lung. Thus, BAL protein content, a sensitive indicator of lung injury, has been standardized in uninjured sheep lung.

Polyacrylamide Gel Electrophoresis of Ovine Bronchoalveolar Lavage: We performed polyacrylamide gel electrophoresis (PAGE) on the five BAL samples obtained from the above studies. Cell free BAL samples were obtained as described above and the sample adjusted to a concentration of

425 µg/ml in sample application buffer (SAB) (0.1M tris-HCl (pH 6.8), 1.0M urea, 2.0% sodium dodecyl sulfate (SDS), 0.002% bromphenol blue, 20% glycerol). Following ETOH precipitation and centrifugation (15,000 x g), protein precipitate was reconstituted in SAB. Samples were denatured by boiling and applied to polyacrylamide gel consisting of 3.5% stacking gel (2.6% total acrylamide as bis, 0.125M tris-HCl, 0.5M urea, 0.1% SDS), and 4.0-10% resolving gel (2.6% total acrylamide as bis, 0.375M tris-HCl (pH 8.9), 0.5M urea, 0.2% SDS). Polymerization was induced by TEMED and ammonium persulfate. Electrophoresis was performed on a Bio-Rad electrophoresis system (Reservoir buffer, 0.192M glycine, 0.025M tris-HCl, pH 8.3) using a Buchler 1-1500 power source. Gels were run at 20 mA until the dye front reached the gel bottom (5-6 hours). Gels were calibrated using prestained standards (Bio-Rad). Gels were stained with xylene green and destained with deionized water.

Figure 20 shows a representative SDS-PAGE from one of the 5 BAL samples obtained. The lanes are displayed in the following order: lane A, molecular weight standards; lane B, 5X concentrated BAL sample from a control, non-injured sheep; lane C, 5X concentrated BAL from a sheep 30 minutes post infusion of ethchlorvynol, lane D, 5x concentrated BAL from the same sheep 1 hour post infusion of ethchlorvynol, lane E, simultaneously sampled sheep plasma from the same animal. This gel demonstrates a number of important findings. First, normal sheep BAL contains low concentrations of molecular weight proteins of as high as 160 kD but no discernible signal detectable above 200 kD. Thirty minutes following the onset of lung injury, proteins with molecular weights greater than 200 kD (approximately 400 kD) appear in the airspaces along with increased concentrations of the 160 kD proteins observed in basal BAL. At one hour, higher concentrations of the 160 kD proteins are clearly present with now proteins in excess of 600 kD becoming visible. When compared to lane E which contains simultaneously harvested plasma these findings suggest that SDS-PAGE of BAL from the sheep is capable of clearly documenting the loss of molecular sieving characteristics of the alveolar capillary membrane following injury to sheep lung. We conclude that this technique is very valuable in determining the severity of alveolar capillary membrane injury providing additional important information which complements the BAL total protein content. We feel that this is a very sensitive technique for the detection of injury following agent exposure.

Analysis of Arachidonate Metabolites by High Pressure Liquid Chromatography: In this section, we report on the progress towards examination of uninjured sheep lung for the generation of arachidonate

metabolites. In these studies the overall focus will be to establish an analysis of basal production of thromboxane B₂, Leukotrienes B₄, C₄, D₄ in plasma, lung lymph, and BAL fluid. Studies using HPLC were begun in April of 1990 and have progressed well. In this report, we will present the results of our studies concerning Leukotriene B₄ analysis in ovine plasma and BAL fluid. Lung lymph samples thus far are not available and will appear in subsequent reports. For a multiplicity of reasons, we are making attempts to use HPLC to quantify AA metabolites in all biological fluids. In the purchase of equipment, we have outfitted the HPLC system with specially designed narrow bore reverse phase C-18 columns to enable us to detect picogram quantities of AA metabolites. Quantifying AA metabolites by HPLC instead of radioimmunoassay systems will be equally as accurate as radioimmunoassay kits. We are additionally adapting the Shimadzu HPLC system through a technique termed "Column Switching" to actually process and extract AA metabolites from unprocessed cell free BAL, plasma, and lung lymph. Column switching for the processing of freshly harvested biological fluids offers the advantage of being able to immediately apply samples obtained from the animal to the HPLC apparatus. This will result in far less loss of biological activity by degradation of the specimen. Until the column switching technique is mastered we will extract AA metabolites as previously outlined (see below).

Extraction Procedure: AA metabolites were extracted from ovine plasma and BAL (n = 5) fluid following 5X concentration over an ultrafiltration membrane. All samples were precipitated in 80% methanol for 12 hrs. Octadecyl (C18) extraction columns in luer fittings of a Baker extraction system manifold were conditioned by passage of methanol followed by phosphate buffered saline. Samples were then applied to the column by negative pressure and the column washed sequentially with 2 ml aliquots of deionized water twice. Arachidonate metabolites were then eluted with 100% methanol and subjected to HPLC.

High Performance Liquid Chromatography: The HPLC system is a Shimadzu LC-6A integrated system consisting of UV detector, sample injector, system controller, three pumps, and a C-18 Octa-Decyl Silane 5µm particle size 4.6mm x 250 mm reverse phase analytical column. The guard column was a C-8 Octa-Decyl Silane packed. For the studies reported, we utilized a binary gradient system consisting initially of 10% water, 90% acetonitrile going to 90% acetonitrile and 10% water over a 20 minute period with a flow of 1 ml/minute for the duration of the run.

In our initial experiments, we have established the proper solvent and gradient conditions for chromatographing leukotriene B₄. Figure 21A demonstrates the chromatogram for Leukotriene B₄ at a concentration of 50 ng. Conditions established for the column indicates a retention time of 26.2 minutes for leukotriene B₄. Figures 21B and 21C display representative chromatograms obtained from sheep plasma and BAL specimens. Although in the initial stages of this work, we find no evidence for the presence of leukotriene B₄ in uninjured sheep lung, current work in progress is focused to determine the sensitivity of our reverse phase column system. The equipment in use should allow the detection approximately 100 pg/ml concentration of this leukotriene.

Conclusions: This portion of the work on the contract began formally in January 1990. In this time frame, we have documented the level of alveolar-capillary membrane permeability in sheep lung and the degree of molecular sieving characteristic of sheep lung during basal conditions. We have additionally demonstrated the loss of molecular sieving characteristics of sheep alveolar capillary membrane following onset of Ethchlorvynol-induced lung injury using carefully calibrated SDS-polyacrylamide gel electrophoresis. Finally, we have begun to calibrate the HPLC system for the determination of AA metabolites which may be present in the injured sheep lung following inhalation injury induced by the agents to be employed in these studies. Work is proceeding towards establishing solvent and gradient conditions for the examination of thromboxane B₂, and leukotrienes C₄, and D₄.

Pulmonary Endothelial Cell Studies: One important potential mechanism of pulmonary edema involves effects of immune and inflammatory mediators and cells on pulmonary endothelium. In order to evaluate this process, studies were undertaken using endothelial cells in culture. An initial goal of these studies was to establish cell lines in culture. The following lines have been established: (1) ovine pulmonary artery endothelium, (2) ovine pulmonary microvascular endothelium, and (3) human umbilical vein endothelium. The ovine pulmonary artery cells were isolated fresh and have been established and propagated; these cells replaced a line originally obtained from Dr. Robert Werrlein (at USAMRICD). Assays performed reveal no differences in monolayer behavior between the two lines. Initial isolates of ovine pulmonary microvascular cells have been obtained, and culture and cloning of the line are proceeding. Primary cultures of human umbilical vein endothelium have been harvested and are being propagated; baseline studies are expected to begin soon. Results presented below were obtained in the ovine pulmonary artery endothelium.

Table 3 summarizes results from cytotoxicity studies performed using various lymph, lymphocyte or neutrophil preparations. The assay was performed according to standard operating procedure (see Materials and Methods). The cytotoxic effects of ovine lung lymph (LL), lung lymph lymphocytes (LLL), peripheral lymphocytes (PL) and polymorphonuclear neutrophils (PMN) against ovine pulmonary artery endothelium were assessed. As shown in Table 3, LL, LLL, and PL from baseline, naive lung exhibited no inherent cytotoxicity against the endothelial cells. Baseline PMN exhibited little cytotoxicity and this was not significantly enhanced by treatment with PMA, an agent which enhances PMN function. Since there was a trend toward enhancement of PMN cytotoxicity by PMA, future studies will focus on a concentration response for PMA, as well as effects of PMA on PL mediated cytotoxicity. Finally, studies will begin in a similar design using the human cell line.

A second line of investigation has focused on the adherence of PMN to ovine pulmonary artery endothelium, since PMN adherence to endothelial cells may be important in many types of pulmonary diseases and pathologies, including adult respiratory distress syndrome (17).

Baseline Adherence Assays: The preliminary studies have been aimed at establishing the conditions for exposure of the effector cells to the stimulus, and initiating investigations into the possible adherence mechanisms.

With phorbol myristate acetate, there is no difference between exposing the polymorphonuclear neutrophils prior to the adherence assay or concomitant with the adherence assay (See Figure 22). Thus, for PMA exposure the stimulus is applied during the adherence assay. However, in the case of lipopolysaccharide (LPS), enhancement of PMN adherence required pre-exposure of the endothelial monolayer to LPS for a minimum of 2 hours (results not shown). PMA stimulation markedly enhanced PMN adherence to endothelium. To determine the potential role of the adhesion complex (CD11/CD18) in PMN-endothelial adherence, assays were also run in the presence of monoclonal antibody 60.3 (a generous gift of Dr. Cairran Walsh) (See Figure 23). Within the dose range tested, PMA did not show a dose-dependent response. However, MoAb 60.3 at 5 ug/ml significantly inhibited baseline, as well as stimulated, PMN adherence. This data is similar to that reported in the literature on human and rabbit cell lines. LPS exposure enhanced PMN adherence to endothelium in a dose-dependent manner, though not to the same degree as seen with PMA stimulation.

MoAb 60.3 again demonstrated an inhibition of PMN adherence to endothelium (See Figure 24).

To summarize results of preliminary adherence studies using endothelial cells: (1) Baseline PMNs exhibit a relatively stable adherence profile. (2) PMA stimulation results in a dramatic increase in PMN adherence to endothelium, even at doses which do not increase the cytotoxic potential of PMNs towards endothelium (see above). (3) Studies with the CD 11/18-specific antibody, MoAb 60.3, demonstrate that this complex plays a role in ovine PMN adherence to endothelium, both at rest and following PMA stimulation. (4) Prolonged exposure (> 120 min) of endothelial monolayers to LPS results in increased PMN adherence. (5) MoAb 60.3 has the ability to inhibit adherence of PMNs to endothelium following LPS exposure, although the explanation for this is not readily apparent.

Our current areas of investigation include: (1) Determining the lowest PMA dose capable of enhancing PMN adherence. (2) Performing adherence assays with ovine lymphocytes. (3) Extending the adherence studies into the human lines. (4) Determining the role of adenosine receptors in modulating ovine PMN adherence.

Baseline Permeability Studies: Preliminary permeability studies have centered on demonstrating the restrictive properties of an intact endothelial monolayer to the passage of albumin (in ul). The intact monolayer demonstrates the ability to restrict the passage of ¹²⁵I-albumin. This capability is totally reversed following exposure to 0.05% trypsin-EDTA (See Figure 25). Incubating the monolayer in the presence of PMA (up to 100 ng/ml) has no effect on endothelial monolayer permeability to albumin (results not shown). Our current areas of investigation on these studies include: (1) Determining the actions of ovine effector cells (baseline and stimulated) on endothelial permeability. (2) Extending the permeability assays into the human cell lines. (3) Modifying the permeability assay to measure native albumin transfer obviating the need for radionuclide. This will simplify the assay when performed in the presence of the vesicants and pyrolysis products in the exposure facility.

Table 1 *

Hemodynamic Changes over a 3 day Period in 10 Control Sheep

	SBP	PAP	PWP	RAP	CO
Day 1	86.1 + 13	13.7 + 3.0	6 + 3	0.2 + .4	4.7 + 1.6
Day 2	87.0 + 12	13.8 + 4.0	6 + 3	1.0 + .5	4.8 + 1.4
Day 3	86.0 + 5	13.5 + 0.7	---	---	5.8 + 3.0

* Data expressed as mean +/- Standard Error of the Mean (SEM), Units of items (see glossary)

Table 2 *

Lymph Flow (QL), Arterial Blood Gases and Cellular Changes
over a 3 day Period in 10 Control Sheep

	QL	pCO ₂	pO ₂	Blood WBC	Lymph WBC
Day 1	1.36 + .3	37 + 3.0	97 + 12	7.3 + 1.6	81 + 33
Day 2	1.3 + .3	38 + 4.4	104 + 12	6.8 + 1.9	81 + 48
Day 3	1.3 + .01	---	---	4.9 + 2.2	101 + 99

* Data expressed as Means +/- SEM, Units of Measure (See Glossary)

Table 3*

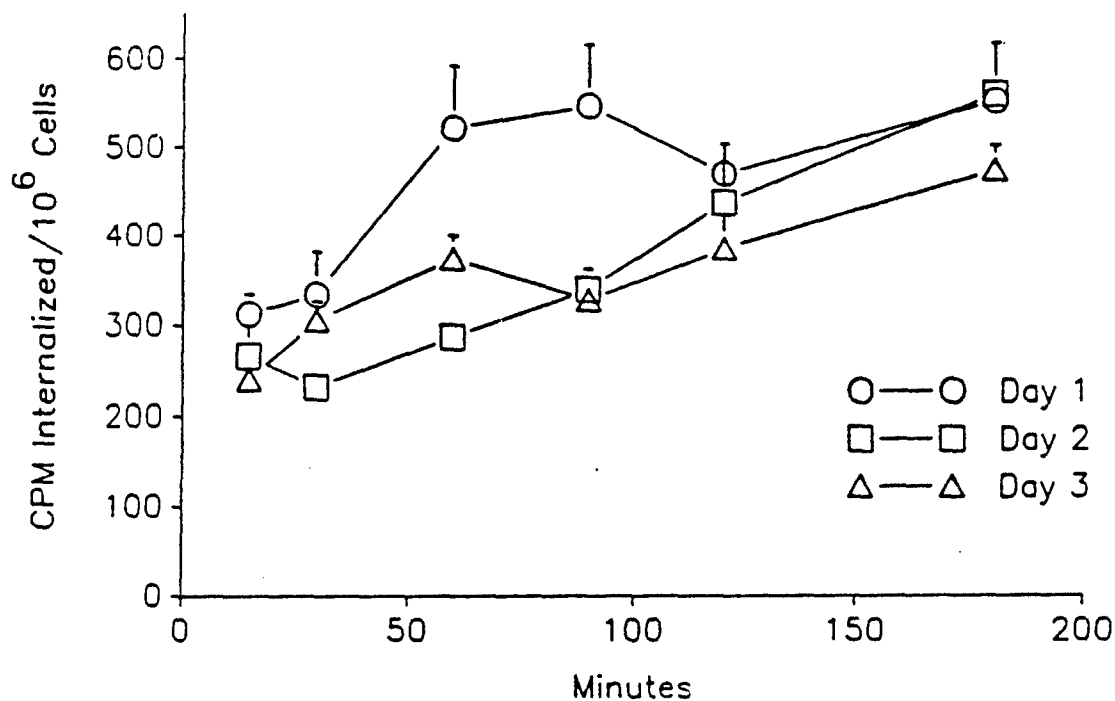
Cytolysis of Ovine Pulmonary Artery Endothelial Cells

Group	(n)	Specific Cytolysis
Baseline Lymph	5	2.07 + 0.98
Baseline LLL (100:1)	5	3.47 + 1.56
Baseline LLL (40:1)	5	2.08 + 1.47
Baseline LLL (10:1)	5	0.08 + 0.04
Baseline LLL (2.5:1)	5	0.43 + 0.10
Baseline PL (100:1)	5	2.00 + 1.02
Baseline PL (40:1)	5	0.20 + 0.12
Baseline PL (10:1)	5	0.75 + 0.24
Baseline PL (2.5:1)	5	2.63 + 1.35
Baseline PMN (100:1)	7	12.85 + 2.56
Baseline PMN (40:1)	7	14.97 + 3.56
Baseline PMN (10:1)	7	7.18 + 1.98
Baseline PMN (2.5:1)	7	6.10 + 2.30
PMN + 1 ng/ml PMA (100:1)	7	18.27 + 4.58
PMN + 1 ng/ml PMA (40:1)	7	22.67 + 6.00
PMN + 1 ng/ml PMA (10:1)	7	12.59 + 3.54
PMN + 1 ng/ml PMA (2.5:1)	7	15.50 + 5.36

* Units Mean +/- SEM

FIGURE 1

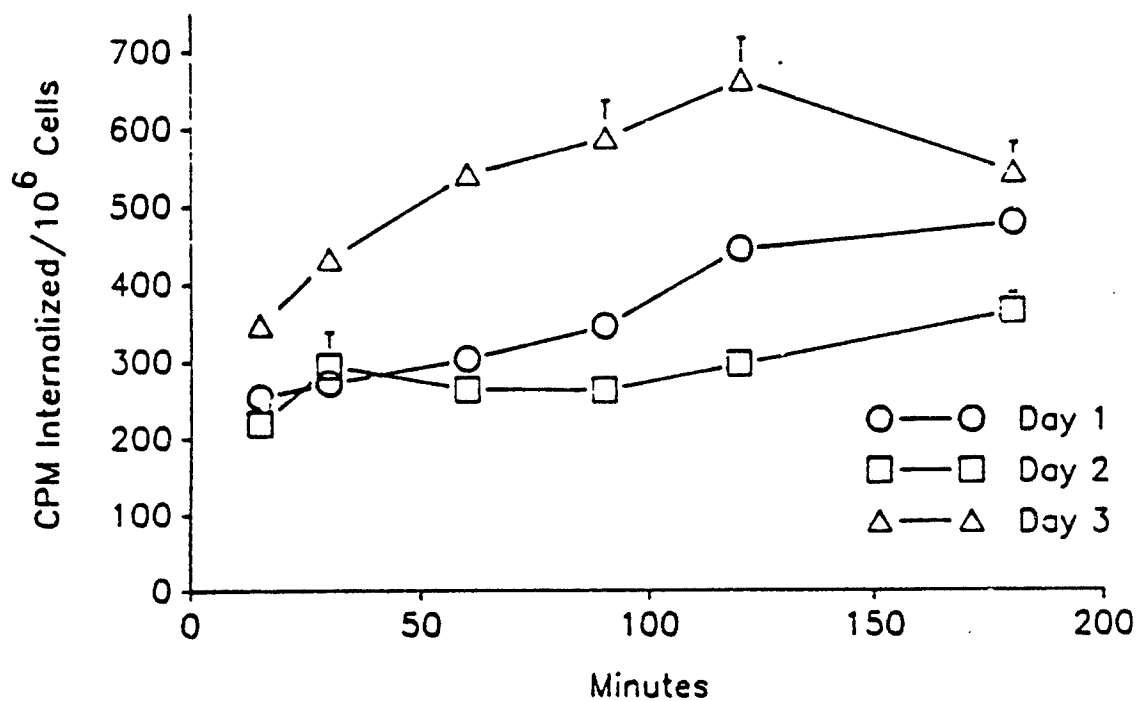
Phagocytosis by Sheep Alveolar Macrophages at 37°C



The phagocytosis of opsonized-⁵¹Cr- sheep erythrocytes by sheep AM was measured over 180 min. Each point is the mean + S.E.M. of 9 experiments. Data shown are from 3 successive lavages of a sheep.

FIGURE 2

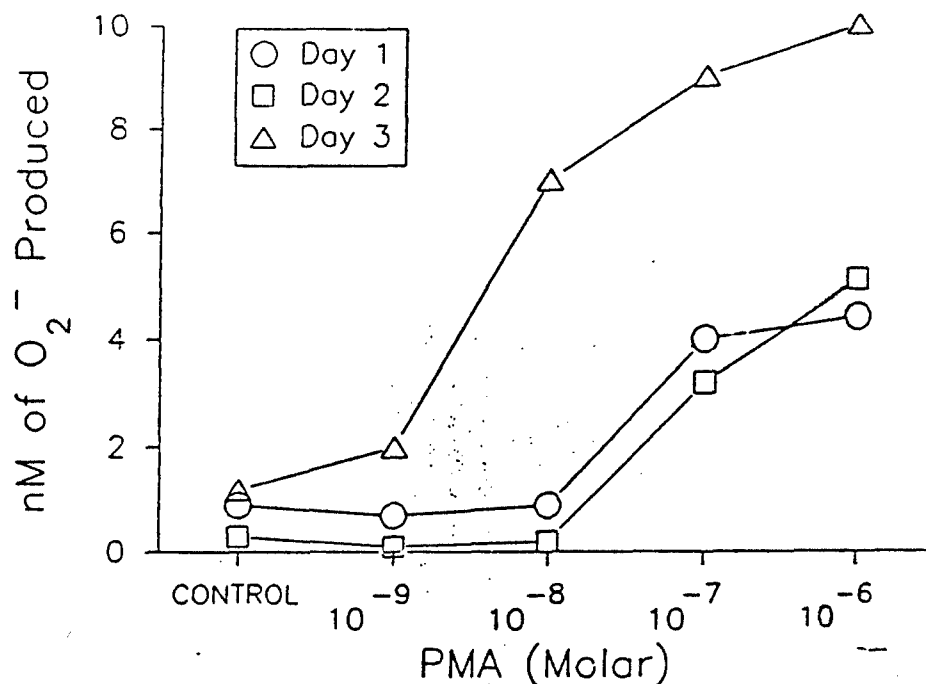
Phagocytosis by Sheep Alveolar Macrophages at 39°C



The phagocytosis of opsonized-⁵¹Cr-sheep erythrocytes by sheep AM was measured over 180 min. Each point is the mean + S.E.M. of 9 experiments. Data shown are from 3 successive lavages of a sheep.

FIGURE 3

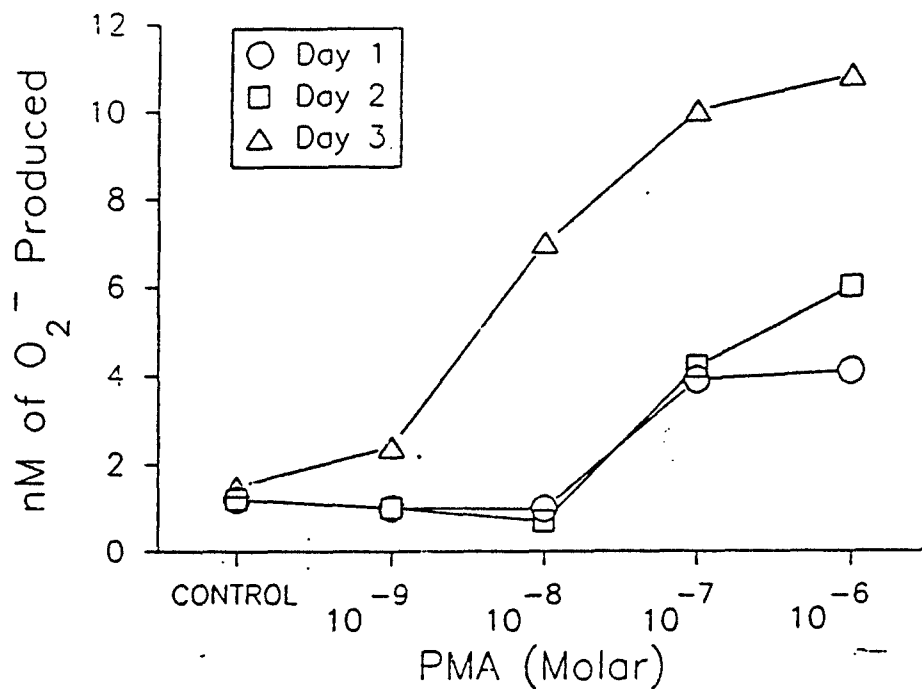
Superoxide Production by Sheep
Alveolar Macrophages at 37°C



Data shown are means of six experiments, each performed in triplicate. Sheep AM were adhered for 1 hour in 24 well Costar plates. Cells were then rinsed and incubated with cytochrome C with and without SOD. Cells were stimulated with PMA at the concentrations shown above. After 90 minutes, the supernatants were aspirated, centrifuged, and absorbance read at 550 nm. Data are expressed as nanomoles of superoxide anion produced.

FIGURE 4

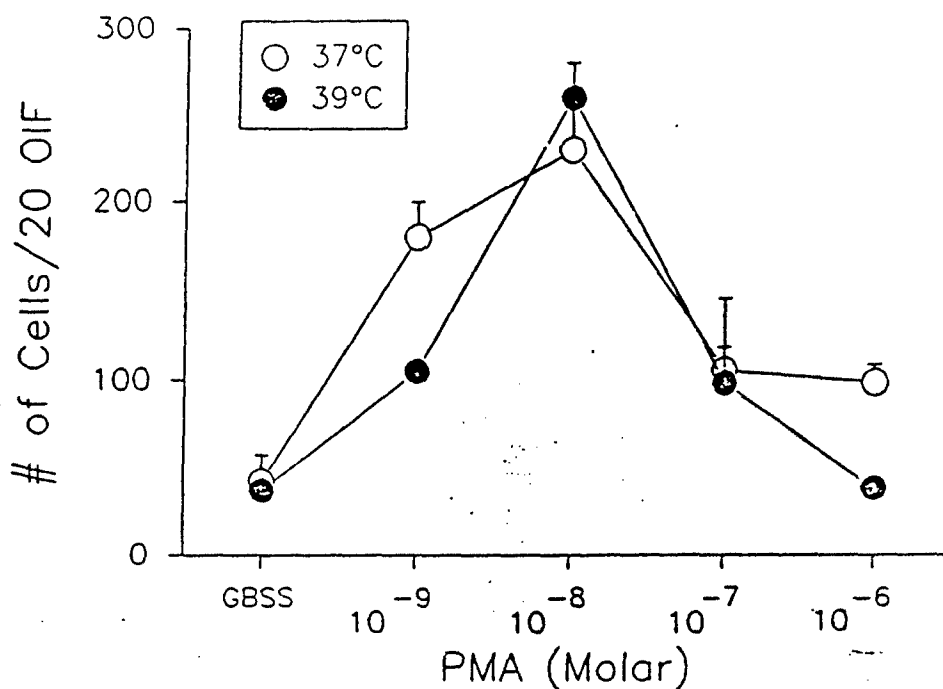
Superoxide Production by Sheep
Alveolar Macrophages at 39°C



Data shown are means of six experiments, each performed in triplicate. Sheep AM were adhered for 1 hour in 24 well Costar plates. Cells were then rinsed and incubated with cytochrome C with and without SOD. Cells were stimulated with PMA at the concentrations shown above. After 90 minutes, the supernatants were aspirated, centrifuged, and absorbance read at 550 nm. Data are expressed as nanomoles of superoxide anion produced.

FIGURE 5

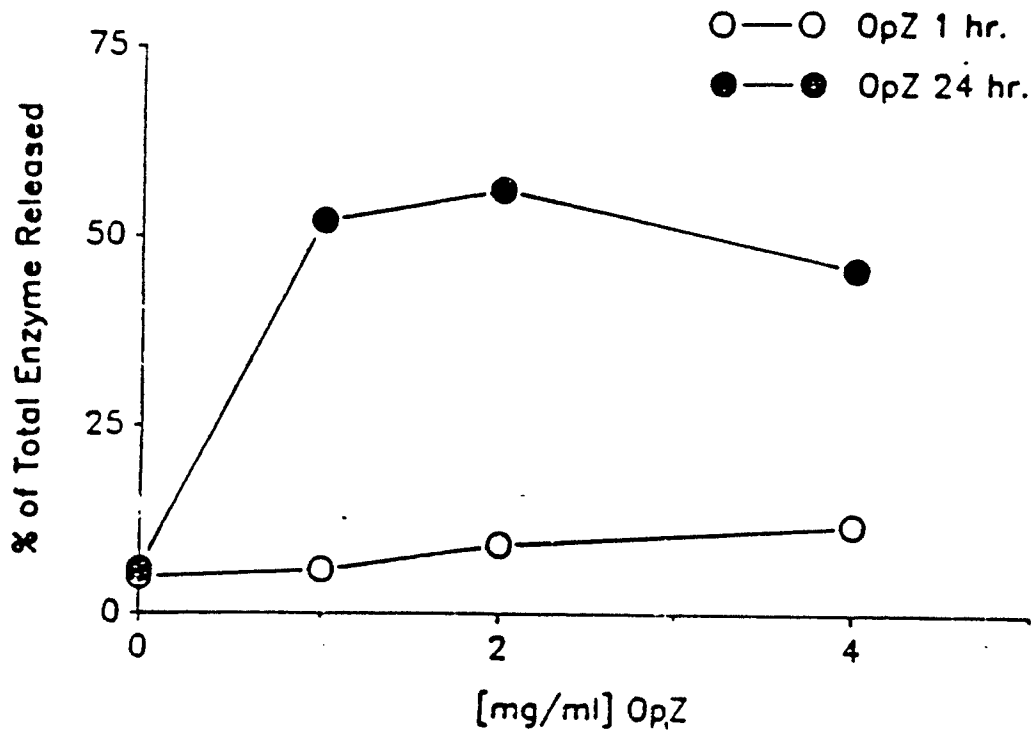
Chemotaxis of Sheep Alveolar Macrophages



Each point represents the mean \pm S.E.M. of six experiments each performed in triplicate. OIF = oil immersion field. Chemotaxis was performed using the Boyden chamber method. Data represents the cells counted that have migrated completely through the filter.

FIGURE 6

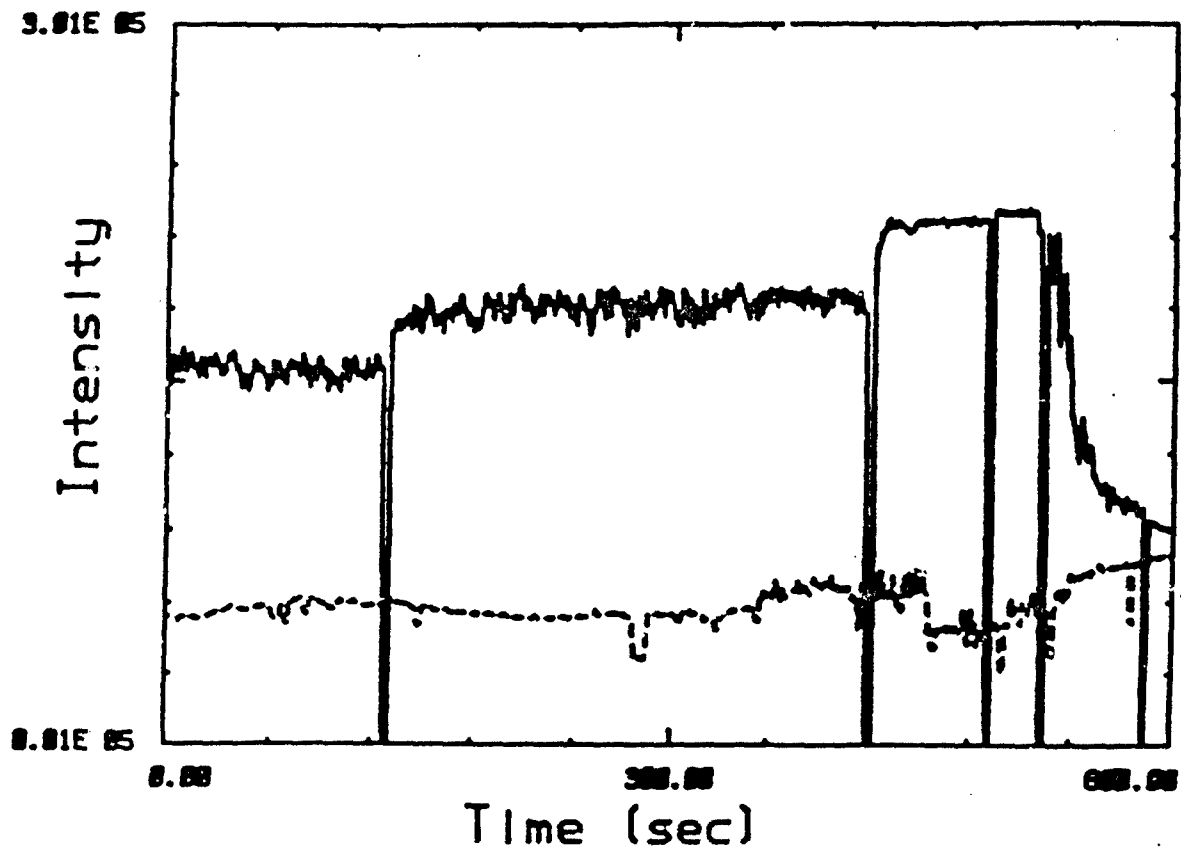
LYSOSOMAL ENZYME RELEASE FROM SHEEP ALVEOLAR MACROPHAGES



Data shown are the means of 6 experiments performed in triplicate. Sheep AM were adhered for 1 hr. in 24 well Costar plates, then OpZ (opsonized zymosan) was added for 1 hr. or 24 hr. as indicated. Supernatants were aspirated and cells were lysed with triton X-100. Samples were assayed for N-acetyl-B-D-glucosaminidase and % enzyme released was calculated.

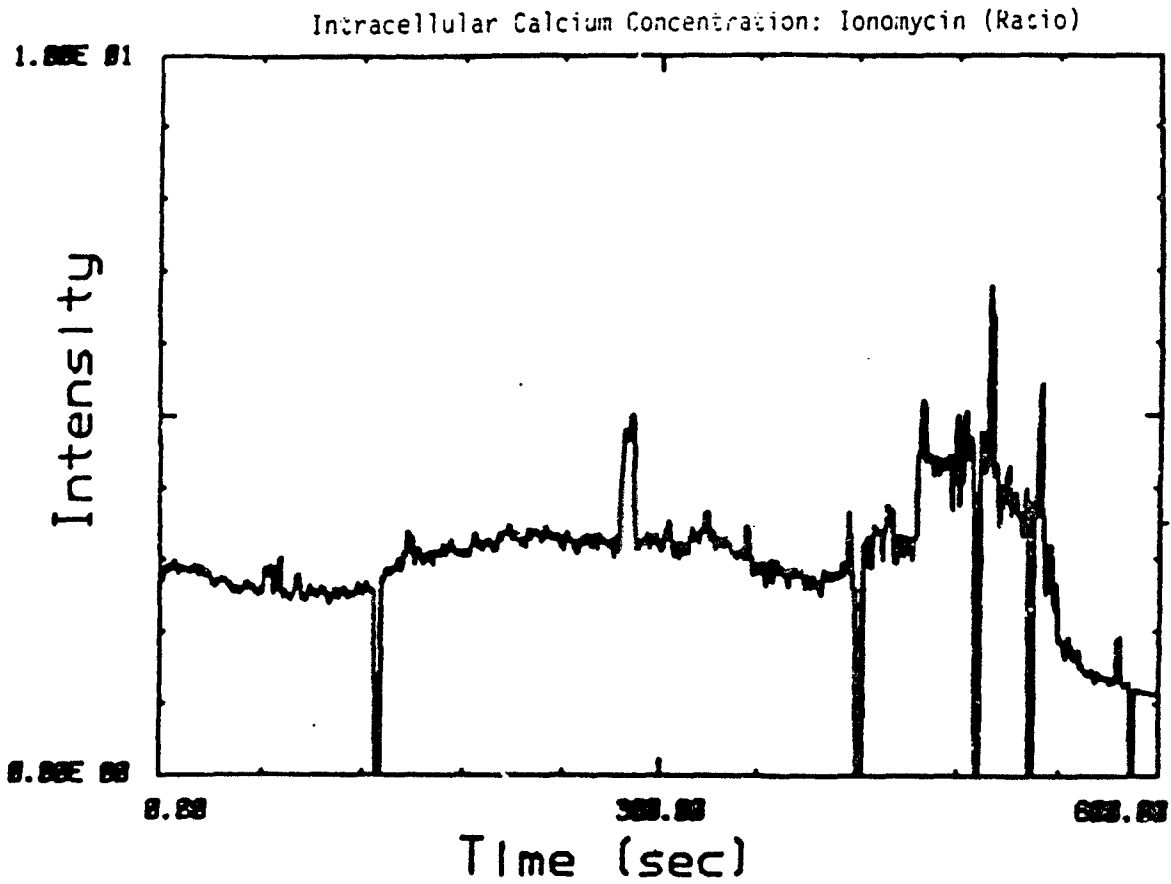
FIGURE 7

Intracellular Calcium Concentration and Ionomycin



Sheep AM were loaded with fura-2/AM for 1 hr. and fluorescence read simultaneously at 340 nm (solid line) and 380 nm (dashed line). Ionomycin (10^{-6} M) was added at 75 seconds. Cells were lysed with triton X-100 at 420 seconds; EGTA was added at 520 seconds. Data are from 1 experiment representative of three performed in duplicate. Data shown represents fluorescence intensity.

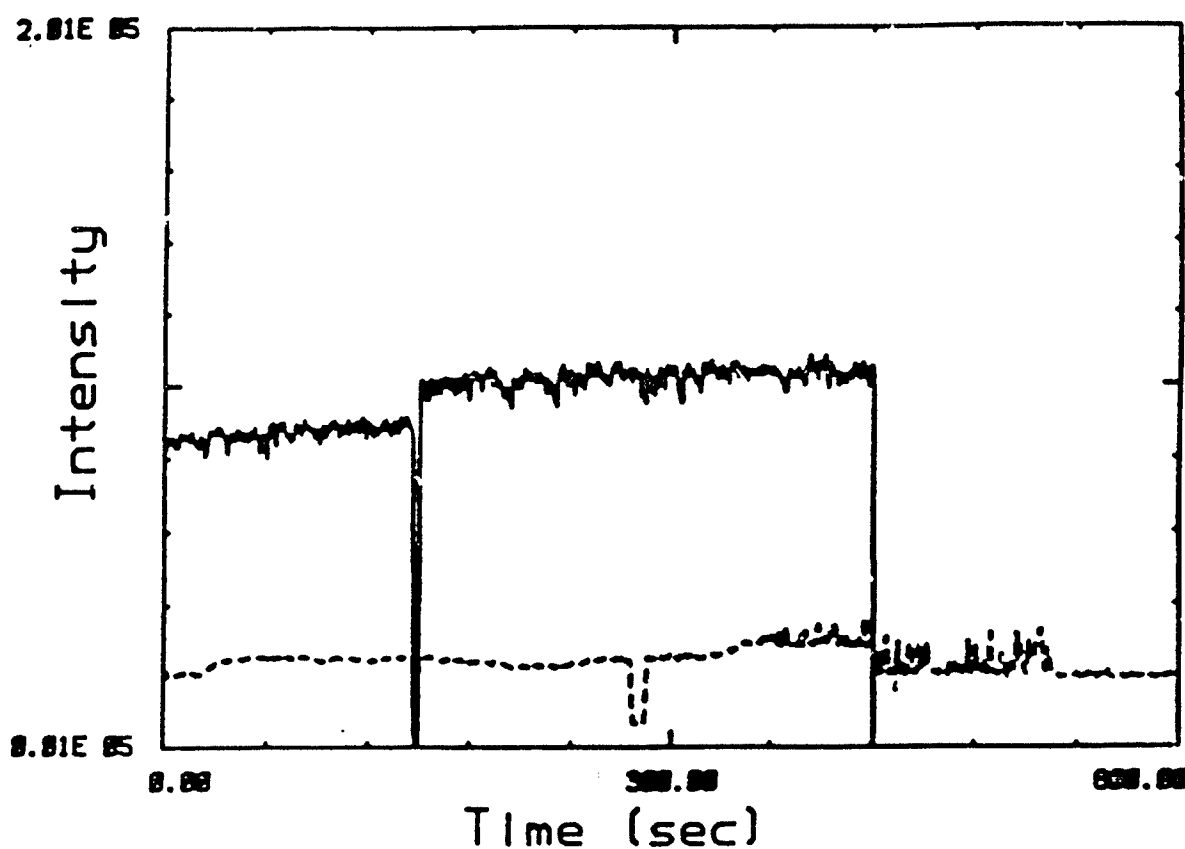
FIGURE 8



Data from Figure 7 are represented as the ratio of 340/380 nm.

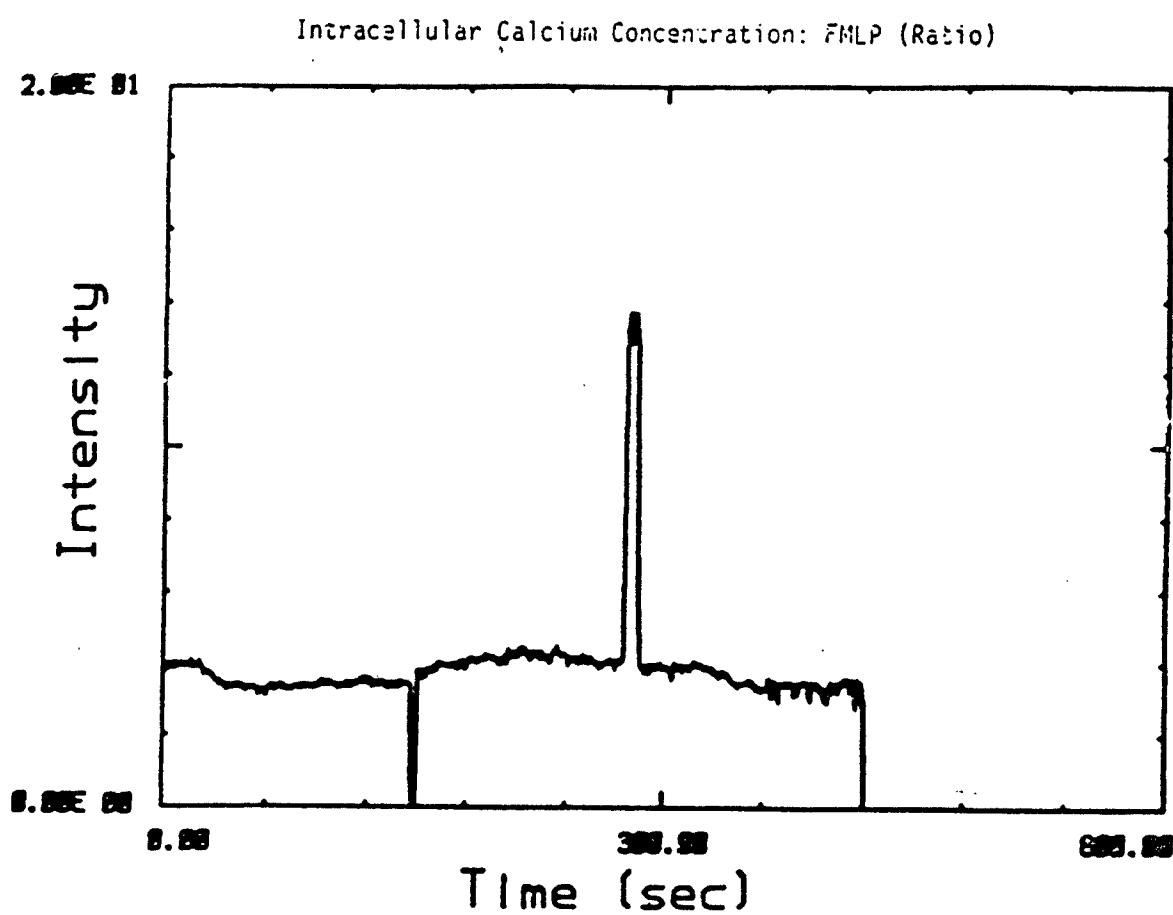
FIGURE 9

Intracellular Calcium Concentration: FMLP



Sheep AM were loaded with fura-2/AM for 1 hr. and fluorescence read simultaneously at 340 nm (solid line) and 380 nm (dashed line). FMLP (10^{-7} M) was added at 150 seconds. Data are from one experiment representative of three performed in duplicate. Data shown represents fluorescence intensity.

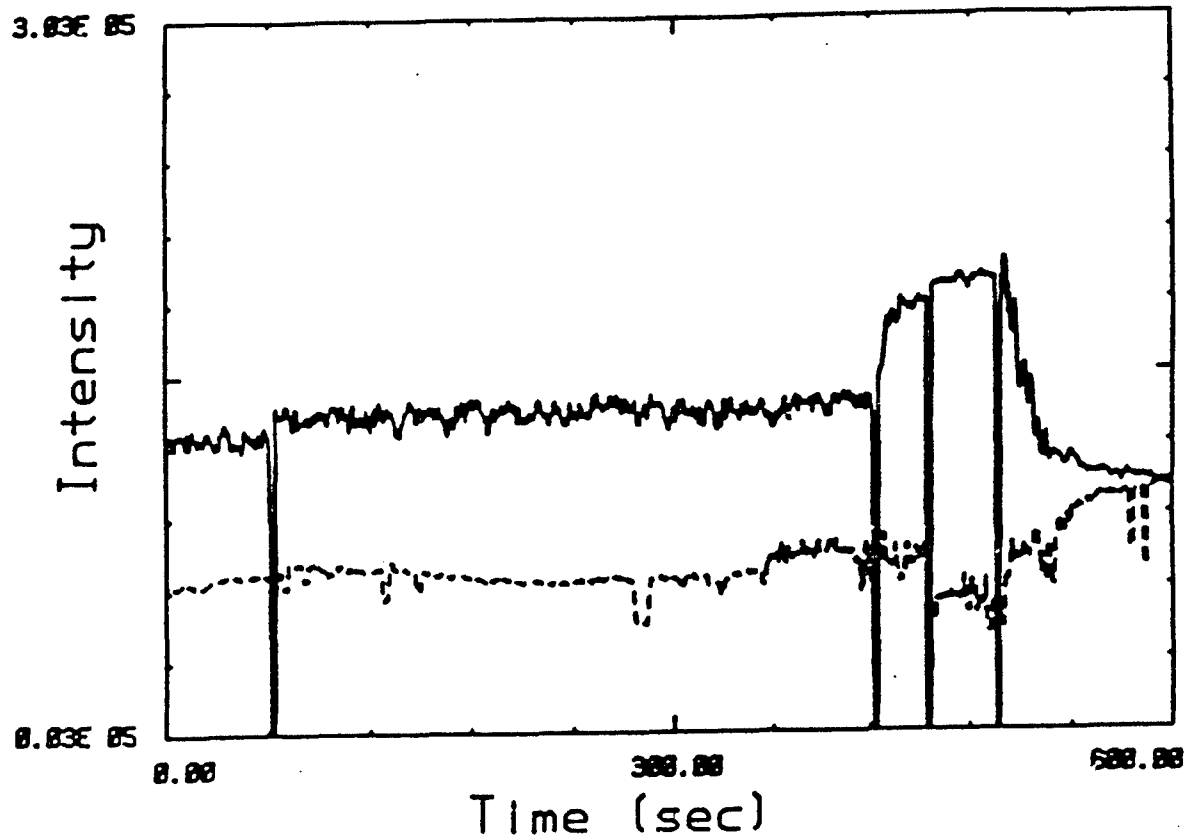
FIGURE 10



Data from Figure 9 are represented as the ratio of 340/380 nm.

FIGURE 11

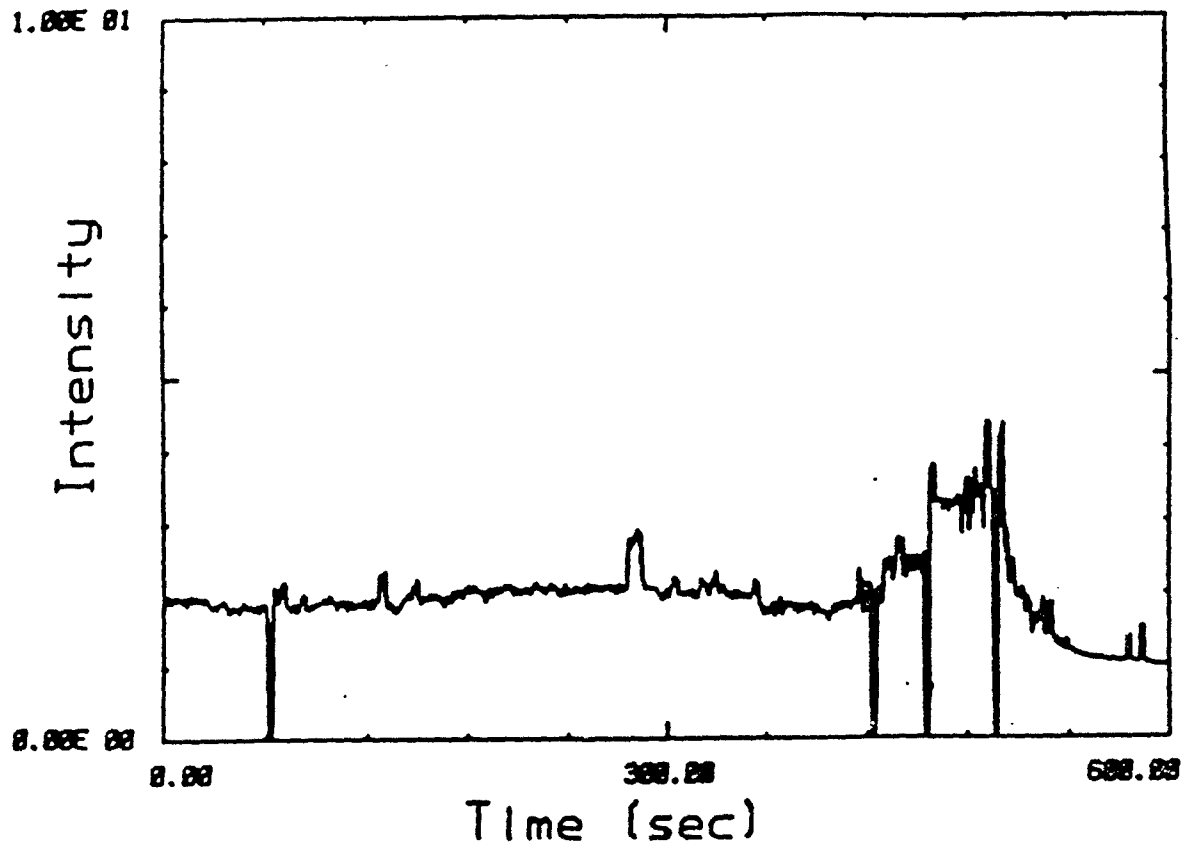
Intracellular Calcium Concentration: PMA



Sheep AM were loaded with fura-2/AM for 1 hr. and fluorescence read simultaneously at 340 (solid line) and 380 nm (dashed line). PMA (10^{-6} M) was added at 60 seconds. Cells were lysed with Triton X-100 at 420 seconds; EGTA was added at 520 seconds. Data are from one experiment representative of three performed in duplicate. Data shown represents fluorescence intensity.

FIGURE 12

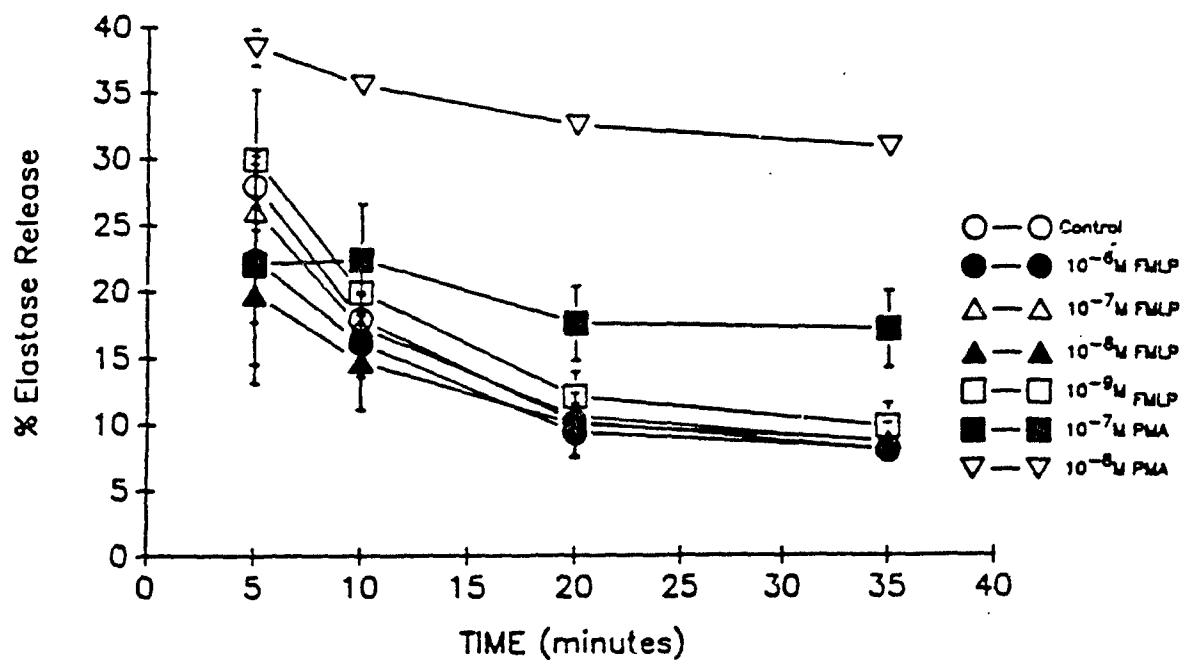
Intracellular Calcium Concentration: PIA (Ratio)



Data from Figure 11 are represented as the ratio of 340/380 nm.

FIGURE 13

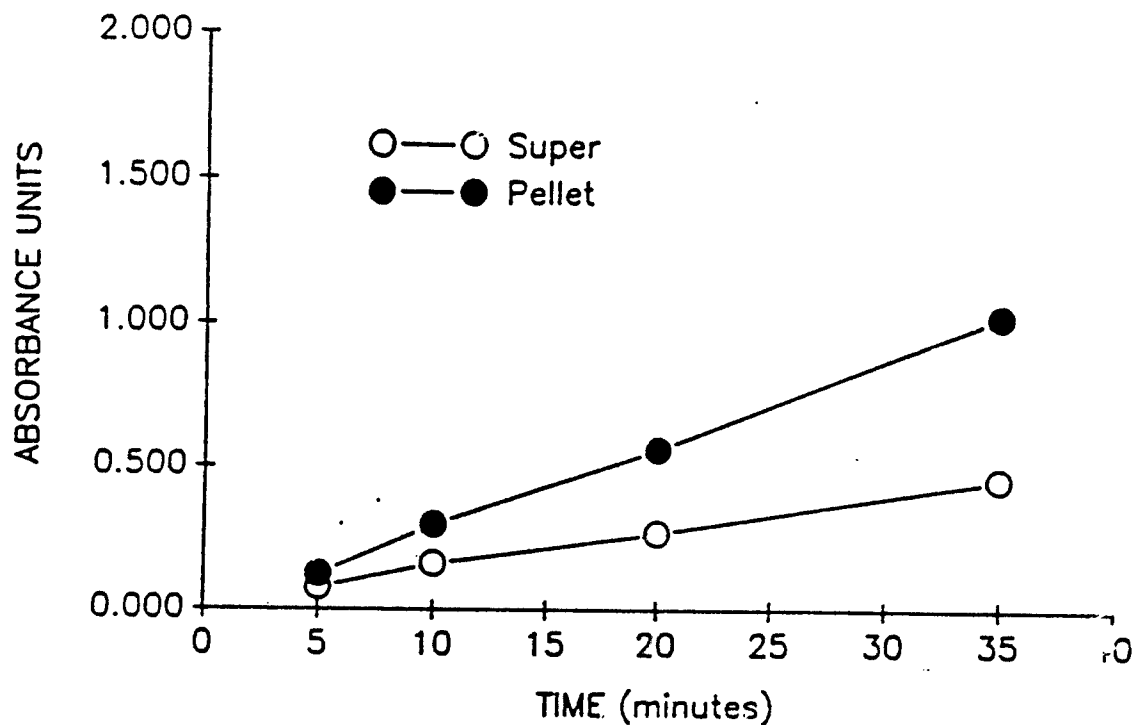
TIME RESPONSE OF HUMAN PMNS ELASTASE RELEASE



The release of the enzyme, elastase, was measured over 35 min. from human PMNs. Each point is the mean \pm S.E.M. of quadruplicate points. The data is representative of duplicate experiments.

FIGURE 14

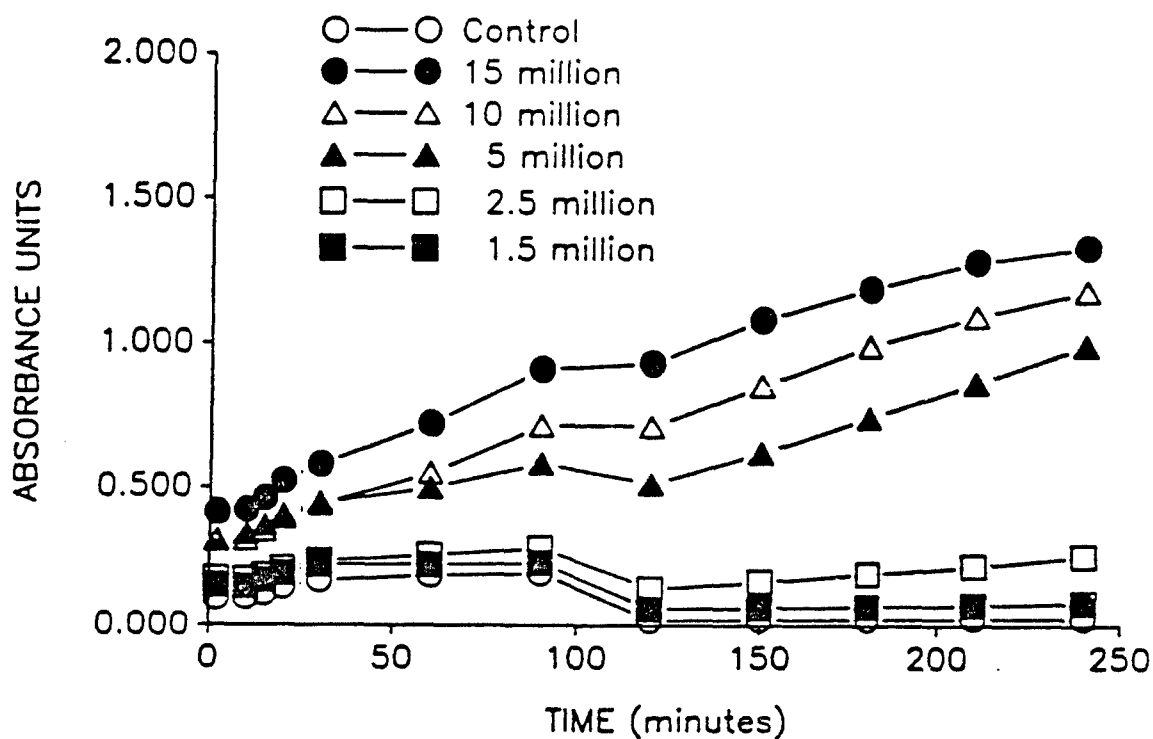
HUMAN PMN ELASTASE ENZYME RELEASE
SUPERNATANT VS. PELLET



The measurement of the supernatant and pellet fraction of human PMNs releasing elastase are shown over time. Each point is the mean + S.E.M. of quadruplicate points. The data are representative of duplicate experiments.

FIGURE 15

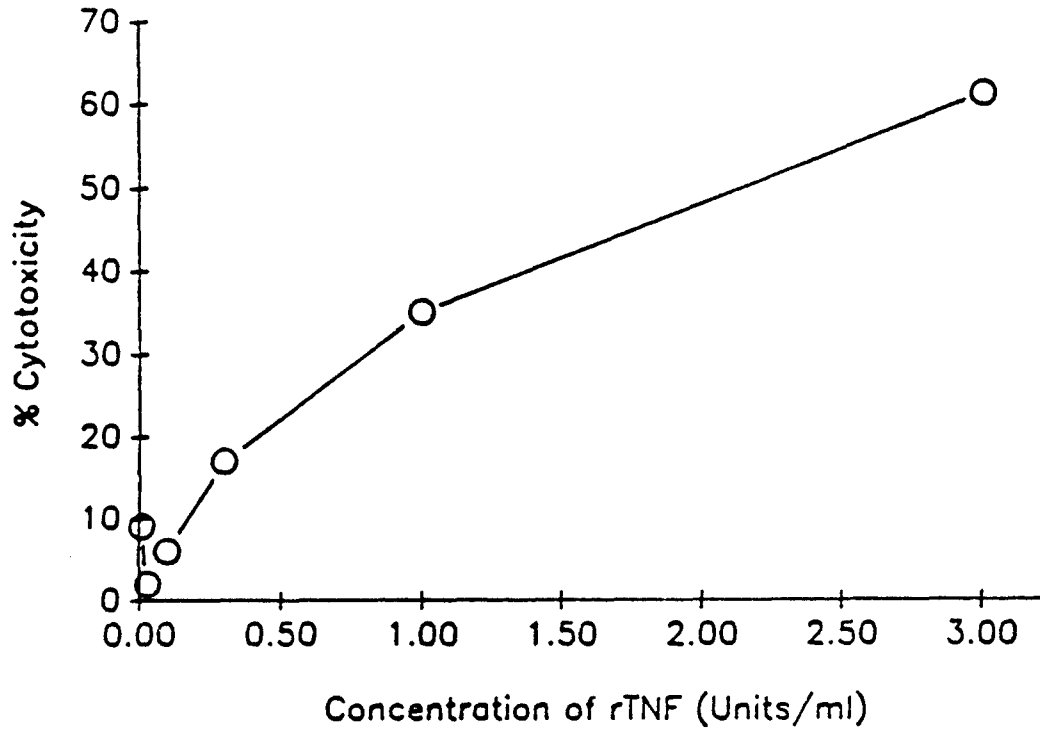
KINETICS OF ELASTASE ACTIVITY IN SHEEP ALVEOLAR MACROPHAGES



Data shown are one experiment representative of two. Sheep AM were aliquoted into the groups delineated above. Cells were washed and the entire pellet lysed with Triton X-100. The elastase assay was performed according to SOP and the development of the enzyme followed over 240 minutes.

FIGURE 16

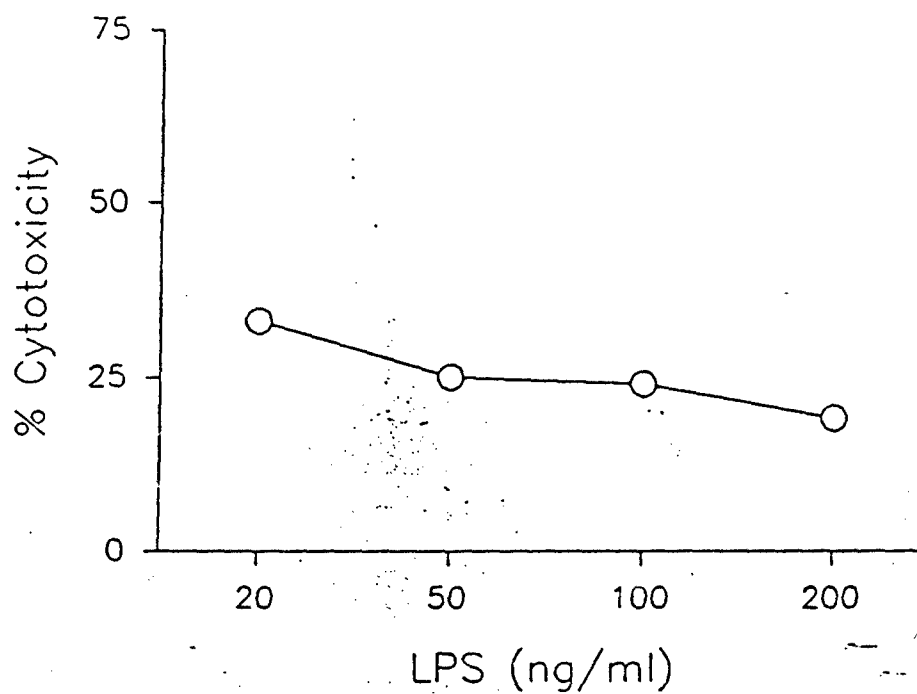
Standard Curve for rTNF Assay



Data shown are from one experiment, representative of 9 experiments. Tumor necrosis factor (TNF) activity was determined by the lysis of L929 cells.

FIGURE 17

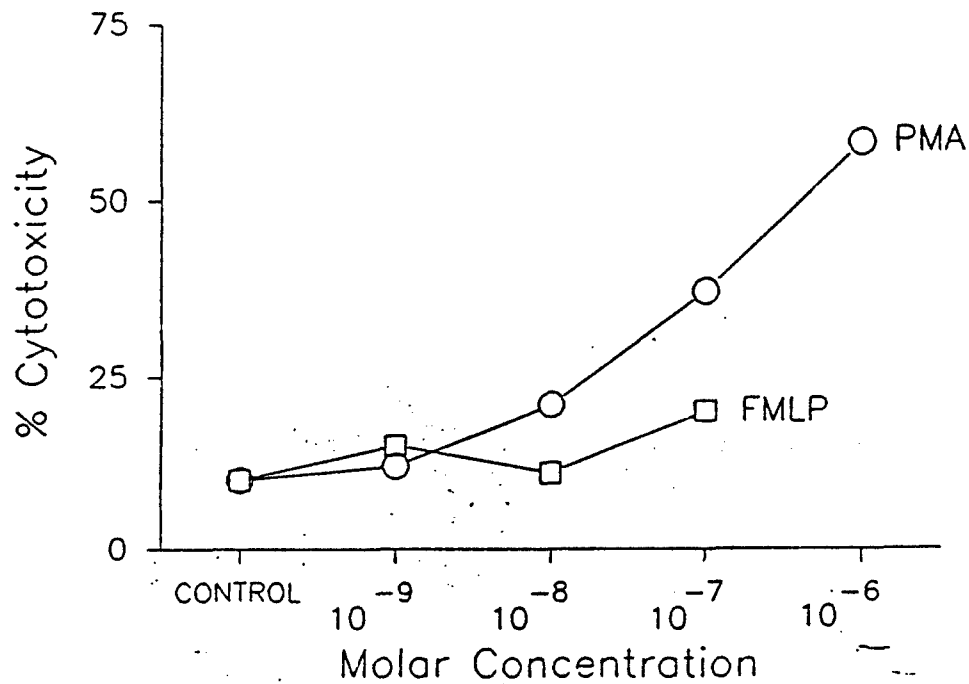
Stimulation of Tumor Necrosis Factor in
Sheep Alveolar Macrophages



Data shown are from one experiment representative of nine experiments.
TNF activity was determined by the lysis of L929 cells stimulated by
lipopolysaccharide (LPS) at the concentrations shown above.

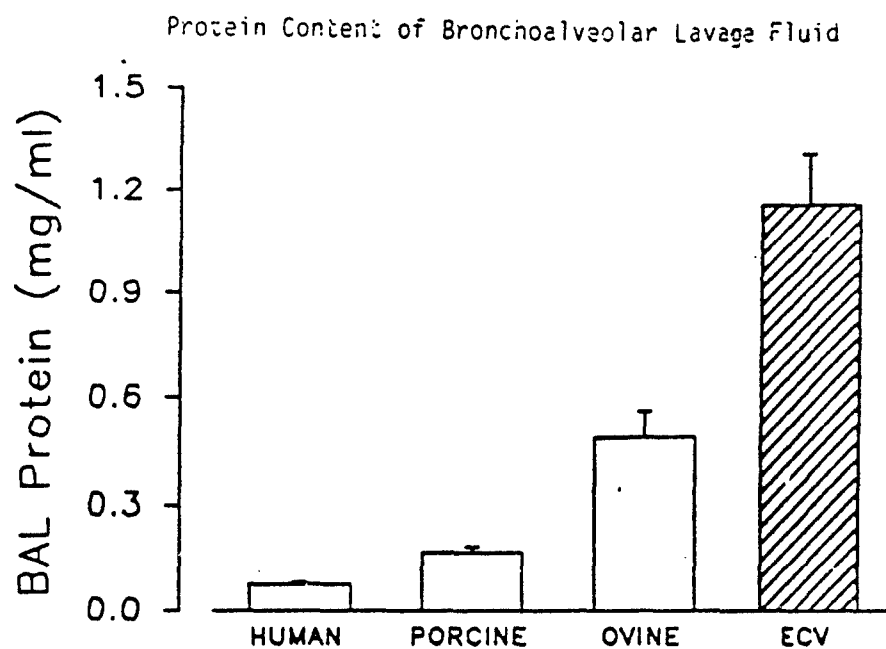
FIGURE 18

Stimulation of Tumor Necrosis Factor in
Sheep Alveolar Macrophages



Data shown are from one experiment representative of nine experiments. TNF activity was determined by the lysis of L929 cells stimulated by either PMA or FMLP at the concentrations shown above.

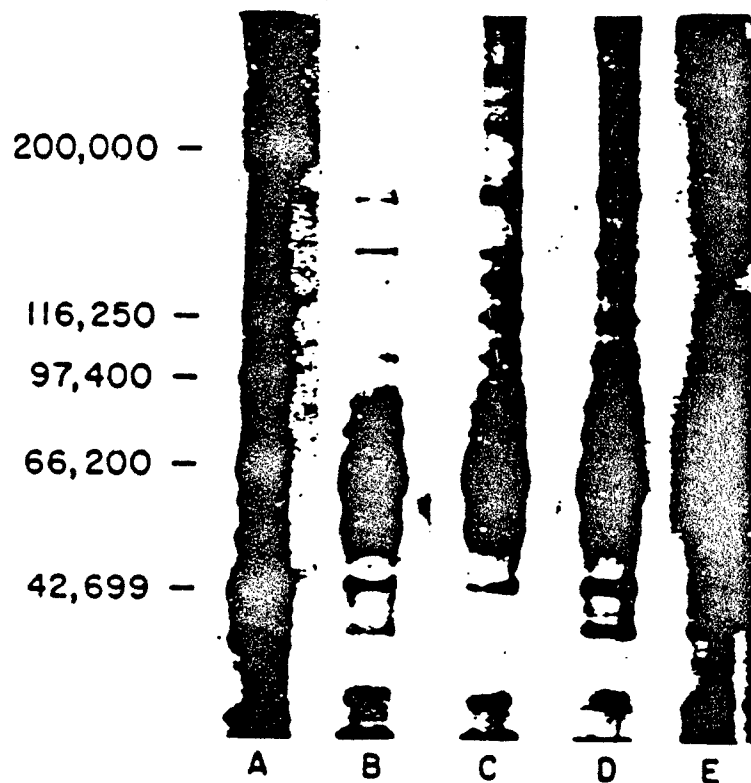
FIGURE 19



Data shown are the mean \pm S.E.M. of 5 experiments each. Lavage fluid was used directly in protein determination assay. ECV (ethchloroviny) group was assayed 1 hr. after treatment of the sheep in vivo with ECV.

FIGURE 20

Polyacrylamide Gel Electrophoresis of Sheep Bronchoalveolar Lavage



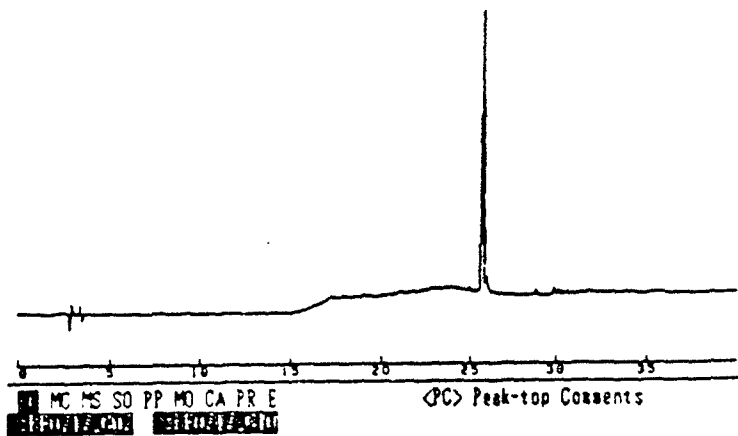
A = SDS molecular weight standards; B = lavage fluid from control, non-injured sheep (concentrated 5X); C = lavage (5X concentrate) from sheep 30 min after treatment with ECV; D = lavage (5X concentrate) from sheep 60 min after treatment with ECV; E = sheep plasma. Samples were applied to SDS-acrylamide gels and run and stained for protein. Y-axis represents molecular weight in daltons.

FIGURE 21

High Performance Liquid Chromatography of Sheep Bronchoalveolar Lavage

BF0719.C01

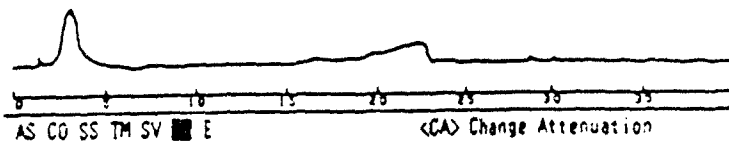
A



B

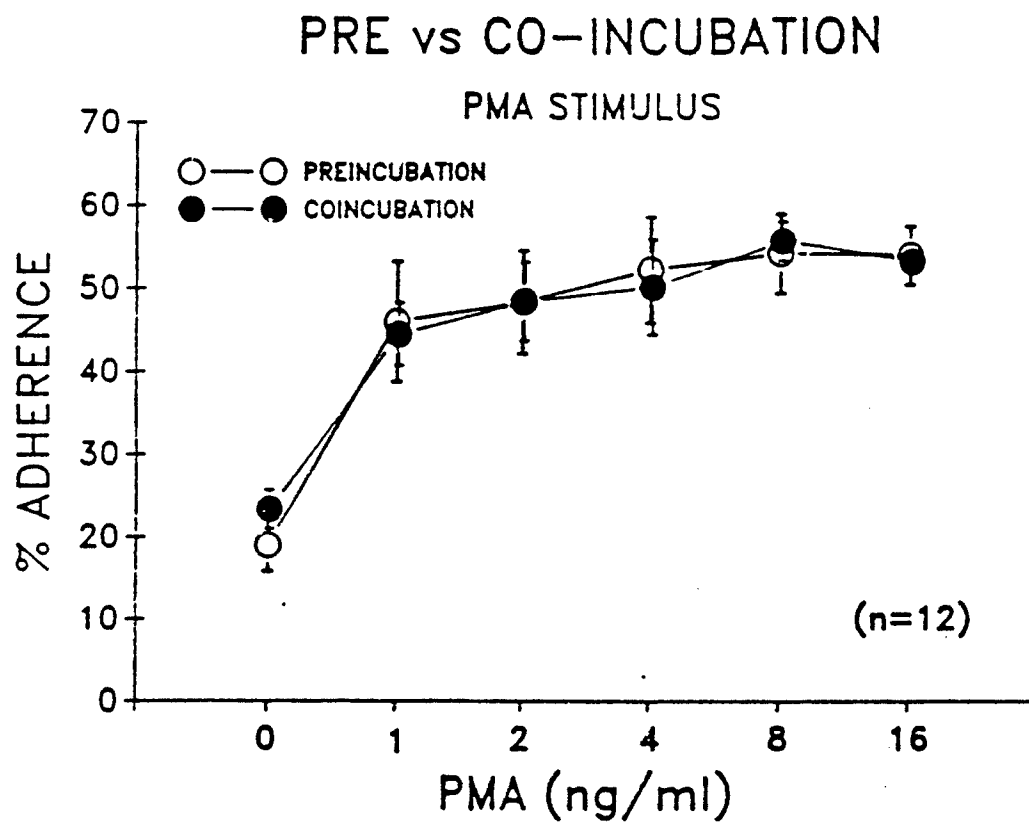


C



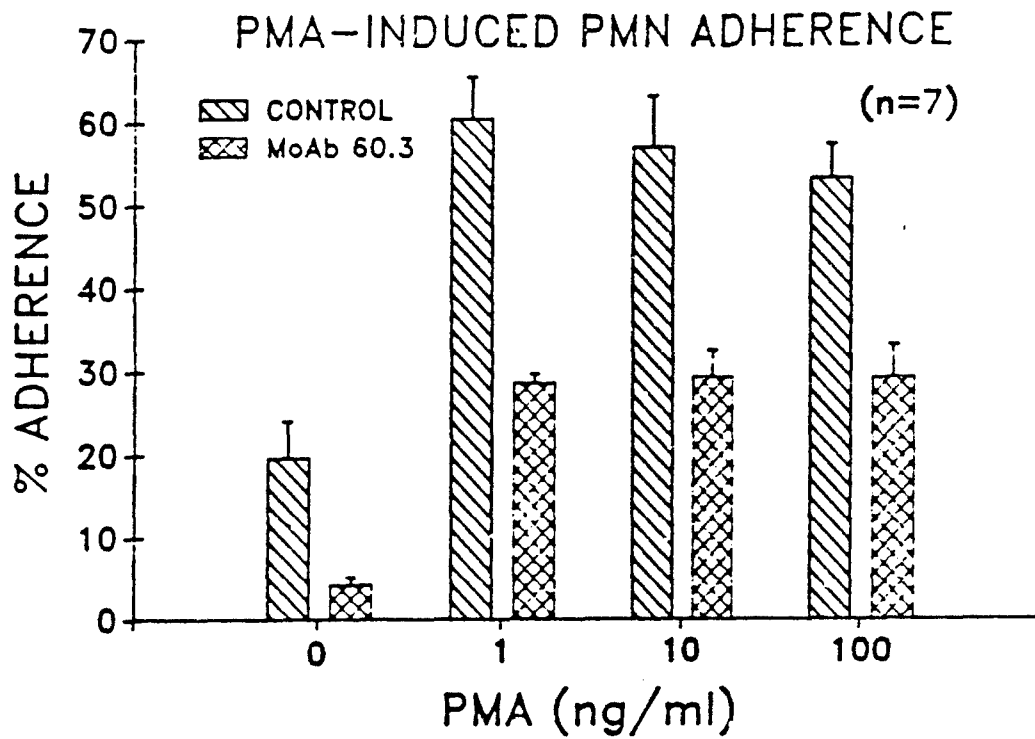
A = 50 ng LTB_4 (leukotriene B_4 standard); B = plasma from control, uninjured sheep; C = BAL from control, non-injured sheep.

FIGURE 22



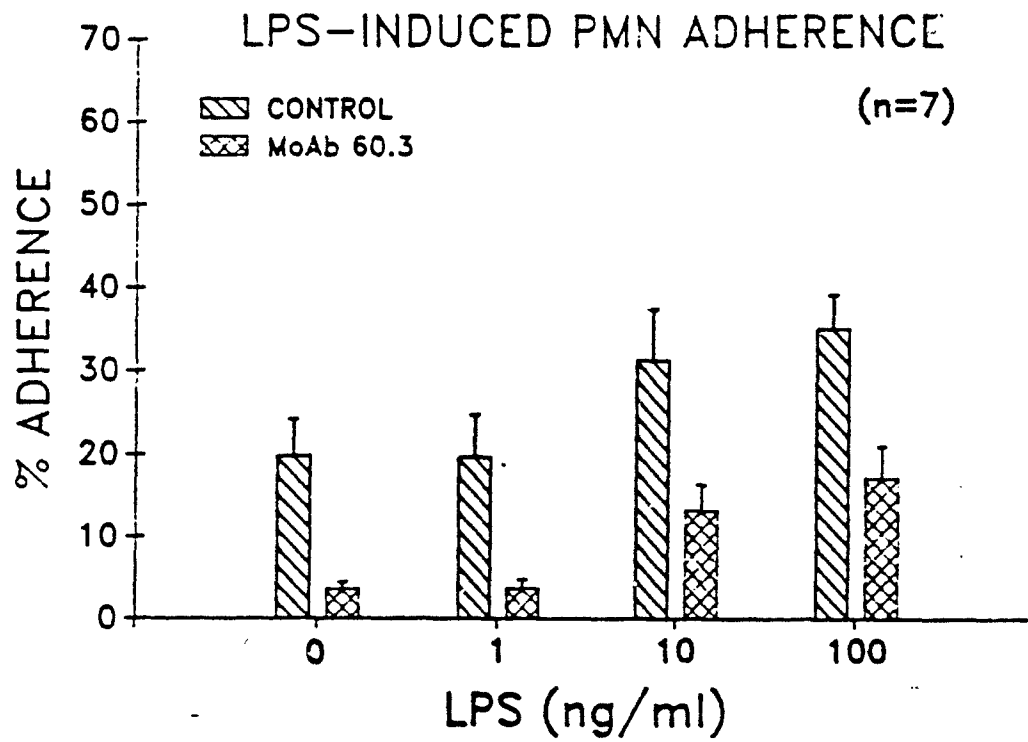
Effect of PMA on adherence of PMN to ovine endothelial cells. Data represent the mean \pm S.E.M. of 12 experiments.

FIGURE 23



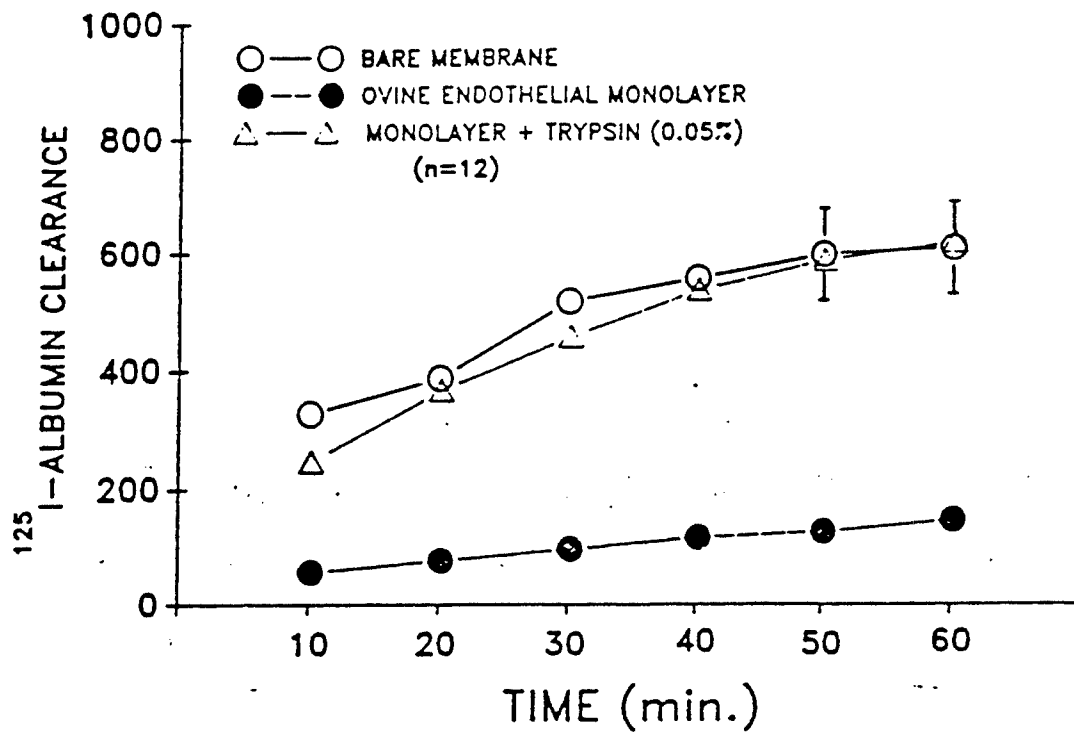
Inhibition of adherence of PMN to ovine endothelial cells by monoclonal antibody (MoAb 60.3). Data represent the mean \pm S.E.M. of 7 experiments.

FIGURE 24



Effect of LPS on adherence of PMN to ovine endothelial cells. Data represent the mean \pm S.E.M. of 7 experiments.

FIGURE 25



Permeability of ovine endothelial monolayer as determined using ^{125}I albumin. Data represent the mean \pm S.E.M. of 12 experiments.

GLOSSARY

AA	Arachidonic Acid
ALB	Albumin
AM	Alveolar Macrophage
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
CaCl ₂	Calcium Chloride
cmH ₂ O	Centimeters of H ₂ O
CO	Cardiac Output (Liters/minute)
CPM	Counts Per Minute
⁵¹ Cr-sRBC	Chromium-labeled Sheep Red Blood Cells
CuSO ₄	Copper Sulfate
DMEM	Dulbecco's Modified Essential Medium
DMSO	Dimethylsulfoxide
ECV	Ethchlorvynol
EGTA	Ethyleneglycol-bis (B-aminoethyl ether) N,N,N',N'-Tetraacetic Acid
EIA	Enzyme Immunoassay
ETOH	Ethanol

FBS	Fetal Bovine Serum
FIO ₂	Fraction of Inspired Oxygen
FMLP	N-Formyl-Methionine-Leucine-Phenylalanine
FOB	Fiberoptic Bronchoscope
Fura-2	Fura-2-acetoxymethylester
GBSS	Gey's Balanced Salt Solution
HBSS	Hanks's Balanced Salt Solution
HEPES	N-2-hydroxyethyl-piperazine-2-ethanesulfonic acid buffer
HPLC	High Pressure Liquid Chromatography
HPMN	Human Polymorphonuclear Leukocytes
¹²⁵ I-albumin	Iodine Labeled Albumin
I.V.	Intravenous
kD	Kilodaltons
LD ₅₀	Concentration that Produces 50% Lethality
LER	Lysosomal Enzyme Release
LL	Lung Lymph
LLL	Lung Lymph Lymphocytes
LPS	Lipopolysaccharide
MgSO ₄	Magnesium Sulfate
mmHg	Millimeters of Mercury
MoAb	Monoclonal Antibody

NA	Nitroanilide
NaCL	Sodium Chloride
Na ₂ CO ₃	Sodium Bicarbonate
NAGA	N-Acetyl-b-D-Glucosaminidase
NaH ₂ PO ₄	Sodium Phosphate
O ₂ -	Oxygen Free Radical
OD	Optical Density
OIF	Oil Immersion Field
OIF	Oil Immersion Field
OpZ	Opsonized Zymosan
PAGE	Polyacrylamide Gel Electrophoresis
PaO ₂	Pulmonary Arterial Oxygen (mm Hg)
PAP	Pulmonary Atrial Pressure (mm Hg)
pCO ₂	Partial Pressure of Carbon Dioxide (mm Hg)
PMA	Phorbol-12-Myristate-13-Acetate
PMN	Polymorphonuclear Neutrophils
pO ₂	Partial Pressure of Oxygen
Ppa	Pulmonary Arterial Pressure
Ppc	Pulmonary Capillary Pressure
PS	Permeability Surface Product
PWP	Pulmonary Wedge Pressure (mm Hg)

QL	Lymph Flow (ml/hour)
RAP	Right Atrial Pressure (mm Hg)
RIA	Radioimmunoassay
RR	Respiratory Rate
rTNF	Recombinant Tumor Necrosis Factor
SAB	Sheep Application Buffer
SAM	Sheep Alveolar Macrophage
SBP	Systemic Blood Pressure (mm Hg)
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SFM	Serum Free Media
SOA	Superoxide Anion
SOD	Superoxide Dismutase
TCA	Trichloroacetic Acid
TNF	Tumor Necrosis Factor
Torr	Partial Pressure of Determined Blood Gas (ie. O ₂ , CO ₂)
TxB ₂	Thromboxane B ₂
V _t	Tidal Volume
WBC	White Blood Cells (Millions/Cubic Microliter)
W/D	Wet/dry ratio
CD11/CD18	Neutrophil adhesion receptor complex

REFERENCES

1. Staub NC, Bland RD and Brigham KL: Preparation of chronic lung lymph fistulas in sheep. *J Surg Res* 19:315-310, 1975.
2. Brigham K, Woolverton WC, Blake LH and Staub NC: Increased sheep lung vascular permeability caused by pseudomonas bacteremia. *J Clin Invest* 54:792-804, 1974.
3. Brigham K, Bowers RE and McKeen CR: Methylprednisolone prevention of increased vascular permeability following endotoxemia in sheep. *J Clin Invest* 68:1250-1258, 1981.
4. Begley CJ, Ogletree ML, Meyrick BO and Brigham KL: Modification of pulmonary responses to endotoxemia in awake sheep by steroidal and non-steroidal anti-inflammatory agents. *Am Rev Respir Dis* 130:1140-1146, 1984.
5. Glauser FL, Fairman RP, Milen G and Falls R: Indomethacin blunts ethchlorvynol induced pulmonary hypertension, but not pulmonary edema. *J Appl Physiol* 53:563-566, 1982.
6. Fairman RP, Glauser FL and Falls R: Increases in lung lymph and albumin clearance with ethchlorvynol. *J Appl Physiol* 50:1151-1155, 1981.
7. Heflin AC and Brigham K: Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. *J Clin Invest* 68:1253-1260, 1981.
8. Gee MH, Perkowski SZ, Tahamont MV and Flynn JT: Arachidonate cyclooxygenase metabolites as mediator of complement initiated injury. *Fed Proc* 44:46-52, 1985.
9. Flick MR, Hoeffel JM and Staub NC: Superoxide dismutase with heparin prevents increased lung vascular permeability during air emboli in sheep. *J Appl Physiol* 55:1284-1291, 1983.

10. Flick MR and Staub NC: Leukocytes are required for increased lung microvascular permeability after microembolization in sheep. *Circ Res* 48:344-351, 1981.
11. Johnson A and Malik AB: Effect of granulocytopenia on extravascular lung water content in microembolization. *Am Rev Respir Dis* 122:561-566, 1980.
12. Havill AM, Gee MH, Washburne JD and Premkumar A: Alpha naphthathiourea produces dose-dependent lung vascular injury in sheep. *Am J Pathol* 243:505-511, 1982.
13. Flick MR, Julian NM and Hueffel JM: Leukocytes are not required for oleic acid injury in sheep. *Physiology* 25:55, 1983.
14. Glauser FL, CeBlois GG, Bechard DE, Merchant RE, Grant AJ, Fowler AA and Fairman RP: Cardiopulmonary effects of recombinant interleukin-2 infusion in sheep. *J Appl Physiol* (Submitted), 1987.
15. Henderson RF: Use of bronchoalveolar lavage to detect lung damage. *EnvHealth Perspect* 56:115-129, 1984.
16. Smith BM, Kessler FK, Carchman SH, Carchman RA: The role of calcium in macrophage chemotaxis to the phorbol ester tumor promoter TPA. *Cell Calcium* 3:503-514, 1982.
17. Fowler AA, Fisher BJ, Centor RM, Carchman RA: Development of adult respiratory distress syndrome: Progressive alteration of neutrophil chemotactic and secretory processes. *Am J Pathol* 116:427-435, 1984.
18. Del Mar EG, Largeman C, Brodrick JW, Fassett M and Geokas MC: Substrate specificity of human pancreatic elastase 2. *Biochem* 19:463-472, 1980.
19. Nakajima K, Powers JC, Ashe BM and Zimmerman M: Mapping the extended substrate binding site of Cathespin G and human leukocyte elastase. *J Biol Chem* 254:4027-4032, 1979.
20. Bieth J, Spiess B and Wermuth CG: The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem Med* 11:350-357, 1974.

21. Johnston RB, Godzik CA and Cohn ZA: Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J Exp Med* 148:115-127, 1978.
22. Joseph M, Tonnel AB, Capron A and Voisin C: Enzyme release and superoxide anion production by human alveolar macrophages stimulated with immunoglobulin E. *Clin Exp Immunol* 40:416-422, 1980.
23. Goodell EM, Bilgin S, Carchman RA: Biochemical characteristics of phagocytes in the P388D1 cell. *Exp Cell Res* 114:57-62, 1978.
24. Kessler FK, Goodell EM, Carchman RA: Perturbation of the phagocytic response in P388D1 cultured macrophages by agents altering cell calcium. *Cell Calcium* 1:181-194, 1980.
25. Grynkiewicz G, Poenie M and Tsien RY: A new generation of Ca^{+2} indicators with greatly improved fluorescent properties. *J Biol Chem* 260:3440-3450, 1985.
26. Tsien, RY, Pozzan T and Rink TJ: Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J Cell Biol* 94:325-334, 1982.
27. Flick DA and Gifford GE: Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J Immunol Methods* 68:167-175, 1984.
28. Mukavitz-Kramer S and Carver ME: Serum-free in vitro bioassay for the detection of tumor necrosis factor. *J Immunol Methods* 93:201-206, 1986.
29. Denizot F and Lang R: Rapid colorimetric assay for cell growth and survival. *J Immunol Methods* 89:271-277, 1986.
30. Berridge MJ: Inositoltrisphosphate and diacylglycerol as second messengers. *Biochem J* 220P:345-360, 1984.
31. Johanson WG and Pierce AK: Effects of elastase, collagenase and papain on structure and function of rat lungs in vitro. *J Clin Invest* 51:288-293, 1972.

32. Kaplan PO, Kuhn C and Pierce JA: The induction of emphysema with elastase. I. The evolution of the lesion and the influence of serum. *J Lab Clin Med* 82:349-356, 1973.
33. Weissmann G, Dukor P and Zurier RB: Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. *Nat New Biol* 231:131-135, 1971.
34. Ackerman NR and Beebe JR: Release of lysosomal enzymes by alveolar mononuclear cells. *Nature* 247:475-477, 1974.
35. Ackerman NR and Beebe JR: Effects of pharmacologic agents on release of lysosomal enzymes from alveolar mononuclear cells. *J Pharm Exper Ther* 193:603-612, 1975.
36. Iro H and Aviado DM: Prevention of pulmonary emphysema in rats by progesterone. *J Pharm Exper Ther* 101:197-204, 1968.
37. Ruff MR, and Gifford GE: Tumor Necrosis Factor. *Lymphokines* 2:235-272, 1981.
38. Old LJ: Tumor necrosis factor (TNF). *Science* 230:630-632, 1985.
39. Beutler B and Cerami A: Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature* 320:584-588, 1986.
40. Urban JL, Shepard HM, Rothstein JL, Sugarman BJ and Schreiber H: Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc Natl Acad Sci USA* 83:5233-5237, 1986.
41. Feinman R, Henriksen-DeStefano D, Tsujimoto M and Vilcek J: Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. *J Immunol* 138:635-640, 1987.
42. Mestan J, Digel W, and Mitnacht S: Antiviral effects of recombinant tumor necrosis factor in vitro. *Nature* 323:816-819, 1986.
43. Kelker, HC, Oppenheim JD, and Stone-Wolff D: Characterization of human tumor necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. *Int J Cancer* 163:632-643, 1986.

44. Matthews, N: Tumor necrosis factor from the rabbit. II Production by monocytes. *Br J Cancer* 38:310-315, 1978.
45. Mannel DN, Moore RN, and Mergenhagen SE: Macrophages as a source of tumoricidal activity (tumor necrotizing factor). *Infect Immun* 30:523-530, 1980.
46. Martinet Y, Yamauchi K and Crystal RG: Differential expression of the tumor necrosis factor/cachetin gene by blood and lung mononuclear phagocytes. *Am Rev Respir Dis* 138:659-665, 1988.
47. Chen AR, McKinnon KP and Koren HS: Lipopolysaccharide (LPS) stimulates fresh human monocytes to lyse actinomycin D-treated WEHI-164 target cells via increased secretion of a monokine similar to tumor necrosis factor. *J Immunol* 135:3978-3987, 1985.

